Docket No.: 13311-00012-US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Agnes Chardonnens et al.

Application No.: 10/523,362 — Confirmation No.: 1864

Filed: February 7, 2005 — Art Unit: 1638

For: NUCLEIC ACID SEQUENCES ENCODING PROTEINS ASSOCIATED WITH ABIOTIC STRESS RESPONSE — Examiner: Vinod Kumar

DECLARATION OF DR. GERHARD RITTE PURSUANT TO 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Gerhard Ritte, a doctor of natural sciences, a citizen of the Federal Republic of Germany and residing at Potsdam, Germany, declare as follows:

1. I am a fully trained biologist, having studied Biology at the University of Göttingen, Germany, from 1989 to 1995. I was awarded my doctor's degree by the University of Potsdam, Germany, in 1999. I worked as an assistant at the department of Plant Physiology at the said University from 1999 to 2007. In 2007, I was awarded the degree doctor rerum naturalium habilitatus by the University of Potsdam.

   I joined Metanomics GmbH, Berlin, Germany, in November 2007, and have since then been working in the field of plant stress physiology.

2. I have read and understood the above-caption patent application (including the current claims), the Official Action dated June 5, 2009, and the references which are relied upon by the US Patent and Trademark Office in the June 5, 2009 Official Action to find that the subject matter being claimed would have been obvious from the "state of the art" at the time of the invention. I have been asked to offer my opinion on whether this invention would have been obvious. For purposes of my analysis, I have been asked to assume that the date of the invention is the earliest filing date claimed by this application, i.e. August

2002. Therefore, my opinion will therefore address the "state of the art" as it existed in August, 2002.

3. I am advised that currently pending claim 1 provides:

> A transgenic plant cell comprising an Oxidoreductase Stress-Related Protein (ORSRP) coding nucleic acid, wherein the nucleic acid comprises a nucleotide sequence encoding a protein having an amino acid sequence with at least 95% identity with the sequence as set forth in SEQ ID NO: 4, wherein expression of said nucleic acid in the plant cell results in increased tolerance to an environmental stress associated with salinity, drought, and/or low temperature as compared to a non-transgenic wild type plant cell of the same species, and wherein the ORSRP is a heat-stable glutaredoxin protein.

The claim as I understand it requires that a nucleic acid encoding SEQ ID NO: 4 – the encoded product of a yeast heat-stable glutaredoxin gene (herein "GRX2 gene" = SEQ ID NO: 4) – is expressed in a transgenic plant cell in order to create a transgenic plant. Expression of the GRX2 nucleic acid allows the creation of transgenic plants which show increased tolerance to salinity, drought, and/or low temperature stress. The patent specification discloses how to make such transgenic plants and the experimental results provided in the specification confirm that increased tolerance to the indicated stresses, relative to control plants, can be achieved when GRX2 is expressed in transgenic plants.

4. As I understand it, the Official Action expresses that a person of ordinary skill in the art in August 2002 would have recognized that expression of a yeast GRX2 nucleic acid in a plant would provide increased tolerance to salinity, drought, and/or low temperature stresses, and that the experimental findings in the specification of the current application which establish this use are not unexpected. Cited to support that conclusion are a published international patent application to *Lanahan et al. ("Lanahan")*[1], and literature references to *Gan*[2], *Grant et al. ("Grant")*[3], and *Samuelson et al. ("Samuelson")*[4].

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1 WO 00/36126.

2 Biochemical and Biophysical Research Communications, 187: 949 (1992).

3 Biochimica and Biophysica Acta, 1490:33 (2000).

4 Plant Physiology, 118:51 (1998).

5. I have reviewed the references cited in the Official Action, and in my opinion, the content of these references is essentially correctly summarized in the Official Action in terms of what those references explicitly disclose. However, I disagree with certain of the conclusions which are drawn from the disclosure of the references in terms of what those references might suggest as to experiments which were not actually performed. In particular, I disagree that a person of ordinary skill working in this art in August 2002 would have extrapolated from these references an expectation that expression of a yeast GRX2 gene in a plant would have resulted in an increased tolerance to salinity, drought, and/or low temperature stresses. A "person of ordinary skill" in this art in August 2002 would have had at least a few years of experience in plant transformation work and familiarity with the scientific literature regarding stress resistance.

6. Initially, I note that there is no demonstration in any of the cited references that expression of a yeast GRX2 gene in a plant confers an increased tolerance to either salinity, drought, and/or low temperature stress in a transgenic plant in relation to a control plant. To the extent such a conclusion can be supported by the references, it is based only upon inference and not upon experimental confirmation. I therefore address whether the skilled person would have expected stress-tolerant utility based on information provided by the cited references, and in particular, whether the skilled person would have found such utility to be expected or predictable.

7. Particularly relevant to whether or not the claimed stress-tolerant phenotype would have been expected or unexpected is the disclosure of *Grant*. Of the cited references, only the *Grant* reference bears directly on this question. The disclosure of *Lanahan* relates to plants containing different proteins (i.e. thioredoxin proteins, which are distinct from glutaredoxin proteins).[5] The reference to *Gan* discloses a relevant nucleic acid sequence (a yeast GRX2 nucleic acid) but discloses no transformation experiments and provides no suggestion as to the expected phenotype of plant cells or plants transformed with yeast GRX2. The *Samuelson* reference

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[5] Although the subject patent application discusses the use of both thioredoxin and glutaredoxin nucleic acids to transform cells and plants, I am advised and it is apparent from claim 1 that the invention as currently being claimed involves transgenic cells and plants expressing the yeast glutaredoxin GRX2.

relates to the expression of yeast iron reductase enzymes.[6] Therefore, I focus my analysis on detailed consideration of the *Grant* disclosure.

8. The background discussion of *Grant* discusses the potential role of yeast glutaredoxins GRX1 and GRX2 in protecting cells against reactive oxygen species (ROS). Page 33, 2d column. It was known at the time of Grant's publication that yeast cells respond to stress conditions by altering expression of "sets of genes" which are mediated by the stress response element (STRE). Page 34, 1st column.

9. The experiments reported by *Grant* investigated the differences in GRX1 and GRX2 gene regulation in response to stress and growth conditions. Page 34, 2d column. Using *lacZ* fusion constructs to determine glutaredoxin expression levels, *Grant* found that expression of both GRX1 and GRX2 increased in response to oxidative stress, heat shock and osmotic shock. Page 35, 2d column. Elevated expression was observed both on glucose media and also using non-fermentable carbon sources. Page 36, 1st column. Yeast cells in exponential phase were challenged with oxidative stress, heat stress and osmotic stress. Page 36, 1st column – page 37, 1st column. Both GRX1 and GRX 2 levels were increased in response to stress, although in different amounts, suggesting their different roles in the cell. Page 37, 1st column. *Grant* then went on to analyze the promoter regions, and identified putative STRE elements associated with the GRX1 and GRX2 promoter regions. The overexpression of GRX1 and GRX2 was associated with a Ras-protein kinase A pathway, an Msn2 and Msn4 transcriptional activation pathway, and the high osmolarity glycerol pathway. Pages 37-39.

10. From these experiments, *Grant* concluded that up-regulation of GRX1 and GRX2 resembled that of many stress-responsive genes. Page 40, 2d column. GRX1 expression was up-regulated significantly more than GRX2 in response to heat and osmotic stress. *Id.*[7]

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[6] *Samuelson* has been cited to show that yeast genes can be successfully expressed in plants to obtain an expected phenotype. Official Action, page 5. The genes encoding iron reductase enzymes used by *Samuelson* are different from the yeast glutaredoxin gene GRX2, and the experiments described by *Samuelson* do not support an unqualified statement that any yeast gene, when expressed in a plant, will provide an expected phenotype in relation to the yeast phenotype.

In my opinion, *Samuelson* would not have led one to predict that expression of yeast GRX2 in plants would confer either expected phenotype or expected enzymatic function.

11. The experiments reported by *Grant* used a GRX2::lacZ fusion construct that contains 933 bp of GRX2 promoter sequence and only a partial coding sequence (44 codons out of the total 109 codons). As identified by *Grant*, the promoter region of the yeast GRX2 gene contains 3 copies of the putative STRE. See page 37, paragraph bridging left and right columns. *Grant*'s experiments confirm that the putative STRE elements found in the promoter region of the GRX2 gene are responsive to the stresses tested. *Grant* provides no experimental evidence that the gene product of the GRX2 gene actually protects yeast cells against the tested stresses, and only a truncated GRX2/lacZ fusion product was expressed.

12. The Official Action states on page 5:

> "It is further maintained that Grant et al. clearly teach that GRX1 and GRX2 (yeast glutaredoxins) are up-regulated by a range of stress conditions including oxidative, heat shock, osmotic (includes salinity) etc. (page 40, 3rd paragraph). It is further maintained that Grant et al. clearly teach that yeast glutaredoxins are small heat-stable oxidoreductases which play an important role in protecting a cell exposed to environmental stresses. Environmental stress would include salt, drought including low temperature. ..."

13. While "environmental stress" in the broad sense of the term would include salinity stress, drought stress, and low temperature stress, *Grant* did not test specifically for any of these particular types of environmental stresses. *Grant* provides no evidence that GRX2 is up-regulated in response to the particular stresses recited in claim 1 (which by their nature are more relevant to growing plants than to growing yeast) or that transgenic GRX2 expression would improve tolerance to these particular plant stresses. While the production of reactive oxygen species (ROS) may be a common feature of different environmental/abiotic stresses, the actual mechanisms of the various stresses, and what pathways they share in common, are not fully understood in either yeast or in plants.

14. It is appreciated by persons working in the art, both now and in August 2002, that the stress response is extremely complex at the biochemical level. As noted at page 2 of the current specification, in relation to plants: "... the cellular processes leading to drought, cold and salt tolerance are complex in nature and involve multiple mechanisms of cellular adaptation and

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[7] To the extent that up-regulation of these genes suggests that over-expression would confer stress tolerance, Grant's results could be taken to suggest that both GRX1 and GRX2 would need to be co-transfected into a recipient host cell for the benefit to be realized.

numerous metabolic pathways." As mentioned above, *Grant* acknowledges the complexity of the stress responses in yeast and refers to activation of "sets of genes" in response to stresses.

15. In particular, at the level of glutaredoxins, a plant system is more complex that the yeast system. In yeast, five (5) glutaredoxin-like proteins are known, including GRX1, GRX2, and a relatively new family identified as glutaredoxin related proteins GRX3, GRX4 and GRX5.[8] Plants contain a higher number of glutaredoxin genes. As of 2004, 31 open reading frames encoding putative glutaredoxin genes had been identified in *Arabidopsis thaliana.*[9] While there might be some amount of redundancy in the functions of the plant glutaredoxins (which is unknown at the present time), the high number of glutaredoxin genes in plants suggests that glutaredoxins in plants have highly specialized functions. The high number of genes also suggests that plant responses functions involving glutaredoxins are more complex and are more intricately controlled than comparable, analogous responses which occur in yeast.

16. At a very fundamental level, plants and yeast are dissimilar in important respects. Yeast are unicellular organisms, whereas higher plants consist of multiple organs which themselves contain various highly specialized cell types. On the single cell level, one prominent difference between plants and yeast is the occurrence of plastids in plants. For example, the plastids of green plant cells, the chloroplasts, are the site of photosynthesis. In contrast, yeast cells do not contain plastids and are not capable of photosynthesis. In addition to the existence of plastids, a plant cell has a more complex endomembrane system. Because the sub-cellular compartments of plant cells can affect the specificity and function of a protein in plants, a gene may perform functions in yeast that it does not normally perform in the plant. Specifically as to glutaredoxin genes, in August 2002 the glutaredoxin genes in plants, and their localization of action inside plant cells, was poorly understood. As stated in one article attached:

> "Grxs are rather well known in bacteria (such as *Escherichia coli*), yeast and mammalian systems. In *E.coli* there are three Grx genes, at least five in yeast and

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[8] See, e.g. Rodriguez-Manzaneque et al., Mol. Cell Biol. 19: 8180 (1999), copy attached as **EXHIBIT 1.**

[9] See, e.g. Rouhier et al., Cell Mol. Life Sci., 2004, 61: 1266-1277, copy attached as **EXHIBIT 2.**

apparently only two in human . . . . On the other hand, the information about plant Grxs is more scarce"[10]

17. In view of the considerations discussed above, I believe that a person skilled in the art in August 2002 would not have had a reasonable basis to believe that expression of a yeast GRX2 gene in a plant would have predictably resulted in an increased tolerance to salinity, drought, and/or low temperature stress. At the most, a skilled person might have recognized a possibility that expression of a yeast GRX2 gene in a plant would have increased tolerance to salinity, drought, and/or low temperature stresses, but this result could not have been confidently predicted.

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I hereby declare that all statements made herein are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Gerhard Ritte

November 4th, 2009
Date

Attachments:

Exhibit 1: Rodriguez-Manzaneque et al., Mol. Cell Biol. 19: 8180 (1999).
Exhibit 2: Rouhier N., et al., Cell Mol. Life Sci., 2004, 61: 1266-1277.
Exhibit 3: Rouhier N., et al., FEBS Letters, 2002, 511: 145-149.

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[10] Rouhier et al., FEBS Letters, 2002, 511: 145-149, at page 145, 2nd column. Copy attached as **EXHIBIT 3.**

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Grx5 Glutaredoxin Plays a Central Role in Protection against Protein Oxidative Damage in *Saccharomyces cerevisiae*

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Glutaredoxins are members of a superfamily of thiol disulfide oxidoreductases involved in maintaining the redox state of target proteins. In *Saccharomyces cerevisiae*, two glutaredoxins (Grx1 and Grx2) containing a cysteine pair at the active site had been characterized as protecting yeast cells against oxidative damage. In this work, another subfamily of yeast glutaredoxins (Grx3, Grx4, and Grx5) that differs from the first in containing a single cysteine residue at the putative active site is described. This trait is also characteristic for a number of glutaredoxins from bacteria to humans, with which the Grx3/4/5 group has extensive homology over two regions. Mutants lacking Grx5 are partially deficient in growth in rich and minimal media and also highly sensitive to oxidative damage caused by menadione and hydrogen peroxide. A significant increase in total protein carbonyl content is constitutively observed in *grx5* cells, and a number of specific proteins, including transketolase, appear to be highly oxidized in this mutant. The synthetic lethality of the *grx5* and *grx2* mutations on one hand and of *grx5* with the *grx3 grx4* combination on the other points to a complex functional relationship among yeast glutaredoxins, with Grx5 playing a specially important role in protection against oxidative stress both during ordinary growth conditions and after externally induced damage. Grx5-deficient mutants are also sensitive to osmotic stress, which indicates a relationship between the two types of stress in yeast cells.

Reactive oxygen compounds, such as hydrogen peroxide, the superoxide anion, and the hydroxyl radical derived from the latter, exert toxic effects on diverse cellular molecules, including the oxidation of protein thiol groups (10). Cells have developed a number of protective mechanisms against this oxidant effect on proteins, the thiol-disulfide oxidoreductase activities of thioredoxins and glutaredoxins being among the more significant of these (9, 17, 18, 34). While thioredoxin directly reduces protein disulfide groups with NADPH as the hydrogen donor, the tripeptide thiol glutathione (L-γ-glutamyl-L-cysteinyl-glycine) in its reduced form (GSH) acts as the hydrogen donor for the reduction of protein disulfides by glutaredoxin (17). It has been proposed elsewhere that thioredoxin and glutaredoxin systems are essential for maintaining the adequate redox state of proteins in the intracellular environment and thus for regulating various cellular activities (1, 9, 17). However, only ribonucleotide reductase and 3′-phosphoadenylylsulfate reductase have been firmly recognized as in vivo targets for both systems (2, 28, 37). Even in this case, not all *Escherichia coli* glutaredoxins seem to participate in protection against oxidation of these substrates (28, 51). Thus, many of the in vivo targets of thioredoxins and glutaredoxins are still to be elucidated (1). Nevertheless, the presence of both thioredoxins and glutaredoxins in different organisms, together with the conservation of their active sites through evolution (17, 18), points to their important role as intracellular protein antioxidants (1).

Two genes encoding glutaredoxins (*GRX1* and *GRX2*) in *Saccharomyces cerevisiae* have been characterized elsewhere (12, 29). Grx2 accounts for most of the glutaredoxin activity during exponential growth (29). The *GRX1* and *GRX2* gene products are highly homologous to rice, pig, and human glutaredoxins, as well as to two *E. coli* glutaredoxins (18, 29). Cell growth is not affected in individual and double *grx1 grx2* mutants in either rich or minimal medium. On the other hand, while *grx1* mutant cells are particularly sensitive to oxidative stress caused by menadione (a generator of superoxide anions), the *grx2* mutant is hypersensitive to hydrogen peroxide (29), suggesting separate roles for Grx1 and Grx2 proteins in protection against several types of oxidative stress. Yeast gluthatione reductase (encoded by *GLR1*) regulates levels of GSH in the cells, providing the substrate for glutaredoxin. Thus, it is also necessary for protection against oxidative stress, as shown by the sensitivity phenotype of *glr1* mutants to reactive oxygen species (15, 42). Yeast mutants with mutations in *GSH1* (which codes for glutathione synthetase) do not grow unless glutathione is added to the medium (54), and diethylmaleate-induced gluthatione depletion causes growth arrest (53). These observations indicate that GSH is necessary for cell proliferation, being required for glutaredoxin-mediated reduction of protein disulfide bonds and/or performing additional essential roles in cell metabolism. With respect to the thioredoxin system in *S. cerevisiae*, neither of the two individual mutants with mutations in the thioredoxin genes (*TRX1* and *TRX2*) presents any defects in cell growth. This contrasts with the case of the double *trx1 trx2* mutant, which grows poorly even though deoxyribonucleotide levels in the cell remain unaltered (40, 41), thus pointing to additional functions of the thioredoxin system besides the role it plays in ribonucleotide reductase activity. The fact that the thioredoxin and glutaredoxin systems display at least partially overlapping functions in maintaining the physiological redox state of yeast proteins is supported by the observation that glutathione reductase func-

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TABLE 1. Strains used in this work[a]

Strain	Relevant genotype	Comments
CML235		Wild type
CML236		Wild type
MML15	*MAT***a** *grx3::kanMX4*	Deletion in CML235
MML16	*MATα grx3::kanMX4*	Deletion in CML236
MML17	*MAT***a** *grx4::kanMX4*	Deletion in CML235
MML18	*MATα grx4::kanMX4*	Deletion in CML236
MML19	*MAT***a** *grx5::kanMX4*	Deletion in CML235
MML20	*MATα grx5::kanMX4*	Deletion in CML236
MML37	*MAT***a** *grx3::kanMX4 grx5::kanMX4*	From a cross, MML15 × MML20
MML39	*MAT***a** *grx4::kanMX4 grx5::kanMX4*	From a cross, MML17 × MML20
MML41	*MAT***a** *grx3::kanMX4 grx4::kanMX4*	From a cross, MML15 × MML18
MML42	*MATα grx3::kanMX4 grx4::kanMX4*	From a cross, MML15 × MML18
MML44	*MAT***a** *grx2::LEU2*	Deletion in CML235
MML45	*MAT***a** *grx2::LEU2 grx3::kanMX4*	From a cross, MML44 × MML16
MML47	*MAT***a** *grx2::LEU2 grx4::kanMX4*	From a cross, MML44 × MML18
MML57	*MATα grx5::kanMX4 tetO$_2$(GRX5)::kanMX4*	From MML20, by transformation with the pCM224 cassette (4)
MML58	*MAT***a** *grx3::kanMX4 grx4::kanMX4 grx5::kanMX4 tetO$_2$(GRX5)::kanMX4*	From a cross, MML41 × MML57
MML59	*MAT***a** *grx2::LEU2 grx3::kanMX4 grx4::kanMX4*	From a cross, MML42 × MML44

[a] Wild-type strains CML235 (*MAT***a** *ura3-52 leu2Δ1 his3Δ200*) and CML236 (*MATα ura3-52 leu2Δ1 his3Δ200*) are spores from FY1679 (diploid, *MAT*a/α *ura3-52/ura3-52 leu2Δ1/+ his3Δ200/+ trp1Δ63/+*; from B. Dujon, Pasteur Institute, Paris, France). The other strains have been obtained during this work.

tion is absolutely necessary for cell growth in aerobic conditions in a *trx1 trx2* mutant background. This is probably due to accumulation of toxic levels of oxidized glutathione in *glr1 trx1 trx2* mutant cells, while the single *glr1* mutant displays normal vegetative growth (42).

Besides thioredoxin and glutaredoxin, other cellular activities protect cells from oxidative damage (19, 38). Some of the responsible genes are under the control of the Yap1 transcription factor (15, 24, 25, 49). However, protection against oxidation is also related to other types of stresses. Thus, the expression of *CTT1* (coding for cytosolic catalase) is induced not only by oxidative damage but also by heat and osmotic stresses, through the action of the Msn2 and Msn4 zinc-finger transcription factors on the STRE elements present in the *CTT1* promoter (32, 33, 47). A number of different mutants which are hypersensitive to oxidative damage also displayed increased sensitivity to osmotic stress (23). One of the mutations in these mutants corresponds to the *SKN7* gene, which codes for a response regulator in a two-component regulatory system that can be activated alternatively by osmotic stress (via the Sln1 phosphorelay) or by oxidative stress and which regulates the expression of a number of genes including the *TRX2* gene coding for a thioredoxin (6, 21, 27, 39). Nevertheless, the molecular basis that explains the relationship between osmotic and oxidative stress still remains to be characterized.

In the course of the *S. cerevisiae* genome sequencing project, a family of three previously unknown open reading frames (ORFs) with homology to glutaredoxin genes has emerged. In this work, we present data confirming that this family of *GRX3*, *GRX4*, and *GRX5* genes code for proteins with glutaredoxin activity and show evidence for a role of these genes in the defense against certain types of stress and for a functional interaction among them and with *GRX2*. Finally, we emphasize the importance of Grx5 in such defense functions.

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains used in this work are described in Table 1. CML235 (*MAT***a** *ura3-52 leu2Δ1 his3Δ200*) and CML236 (like CML236 but *MATα*) were employed as wild-type strains. *E. coli* DH5α was used as a host for DNA cloning. Yeast cells were grown at 30°C in yeast extract-peptone-dextrose (YPD) medium or, when indicated, in SD minimal medium with adequate auxotrophic supplements (3) and glucose (at a 2% concentration) or glycerol (at 3%) as a carbon source.

Gene disruptions and other genetic methods. Standard methods (3) were used for plasmid DNA preparation and manipulation and also for bacterial transformations. Crosses between yeast strains, sporulation, and tetrad analyses were carried out as described in reference 20.

To delete *GRX3*, *GRX4*, or *GRX5* in the wild-type CML235 and CML236 strains, we made use of the *kanMX4* cassette from pFA6a-kanMX4, according to the short flanking homology strategy (52). A similar approach was used for disrupting *GRX2* with *LEU2* as a marker, except that a pFA6a-kanMX4 derivative (plasmid pCM376, containing the yeast *LEU2* gene and flanking regions instead of *kanMX4*) was used for amplification of the disruption cassette. In all cases, the DNA cassette was amplified by PCR with Expand High-Fidelity enzyme (Boehringer), followed by DNA transformation of yeast cells (4). Oligonucleotides for cassette amplification were designed in such a way that most of the targeted gene was disrupted upon transformation with the amplified DNA. Thus, for the disrupted *GRX2* gene, only 9 bp of the original ORF remains at the 5′ end and 11 bp remains at the 3′ end; for *GRX3*, 2 and 19 bp remain, respectively; and for *GRX4*, 10 and 9 bp remain, respectively. For *GRX5*, the deletion covers from base +25 (origin at +1) up to the stop codon. Deletions were confirmed by PCR.

Sensitivity to stress conditions. Exponentially growing cells at about 10^7 cells per ml were treated with the respective compound, which was directly added to the growth medium at the concentrations and during the intervals indicated for each experiment. Untreated cultures were incubated in parallel over the same periods. Viability was determined by colony counts on YPD plates (each dilution three times) after 3 days of incubation at 30°C. Total cell number was determined from formaldehyde-fixed samples, by using an Epics XL flow cytometer (Coulter).

Analysis of cell wall-altered phenotype. Agents used as indicators for cell wall alterations were tested by spotting 4-μl samples of 1/8 serial dilutions of cultures exponentially grown in YPD at 30°C (initial concentration of 10^7 cells per ml) on YPD plates containing the respective agent and monitoring growth after 3 days at 30°C. The following compounds and concentration ranges were employed: calcofluor white, 25 to 75 μg/ml; sodium dodecyl sulfate (SDS), 0.05 to 0.2%; and caffeine, 5 to 20 mM.

Northern blot analyses. RNA purification, electrophoresis, probe labelling with digoxigenin, hybridization, and signal detection were carried out as previously described (11). Signals were quantified with the Lumi-Imager equipment (Boehringer) software. Probes for the *GRX3*, *GRX4*, and *GRX5* genes were generated by PCR from genomic DNA, by using oligonucleotides designed to amplify fragments covering the entire ORF without adjacent sequences.

Preparation of cell extracts and determination of enzyme activities. Extracts were prepared from yeast cells exponentially growing in YPD medium at 30°C by collecting, washing, and finally resuspending them (at 1:100 of the original volume) in 20 mM imidazole buffer (pH 7.0) plus 2 mM EDTA and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.2 mM tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK], and 2 μM pepstatin, final concentrations). Cells were broken by repeated vortexing in cold conditions with an equivalent volume of glass beads (0.6-mm diameter; Sigma), followed by low-speed centrifugation (4,000 × *g* for 5 min at 4°C). This supernatant was again centrifuged at

30,000 × *g* for 40 min at 4°C, and the final supernatant was kept for further analyses.

Glutaredoxin (GSH disulfide oxidoreductase) activity was measured by the reduction of the mixed disulfide formed between β-hydroxyethyl disulfide and glutathione, according to reference 18. Cell extracts were heated at 85°C for 5 min to inactivate glutathione reductase, thioredoxin reductase, and other interfering activities. Glutathione reductase activity was determined as previously described (14), following the decrease in absorbance (340 nm) due to the oxidation of NADPH. Transketolase activity was determined as described in reference 22. The protein concentration was measured by the Bradford method.

Quantification of protein carbonyl groups. The protein carbonyl content in crude extracts was determined according to the dinitrophenylhydrazine derivatization method (26). Quantification was carried out with a Zorbax GF-250 high-pressure liquid chromatography gel filtration column at flow rate of 1 ml/min at 30°C. Absorbance at 276 and 370 nm was monitored with a Waters 996 diode array detector.

Other analytical and preparative protein methods. Analytical SDS-polyacrylamide gel electrophoresis and immunodetection of peptides bound to 2,4-dinitrophenylhydrazones (DNPs) were carried out as previously described (48). Anti-DNP antibodies (supplied by DAKO) were employed at a 1:4,000 dilution. Preparative electrophoresis, peptide mapping (after limited proteolysis with endoproteinase V8 from *Staphylococcus aureus*), and sequencing were conducted as described in reference 50.

Sequence analyses. FASTA analysis (as provided by the Munich Information Centre for Protein Sequences [35]) was initially carried out to compare each pair of protein sequences. Multiple protein alignments were calculated with the ClustalW package (16). The Sequence Space algorithm (8) was applied to regions where significant alignments could be established, in order to classify sequences into groups according to their similarity. The original algorithm was implemented in a Mathematica package, and all computations were performed with this program.

RESULTS

A new glutaredoxin family in *S. cerevisiae*. Analysis of the yeast genome revealed the existence of a family of three ORFs (YDR098c, YER174c, and YPL059w) whose products display significant homology to known glutaredoxins. ORFs YDR098c and YER174c have N-terminal extensions that are absent in YPL059w (Fig. 1A). In the homologous region, the predicted protein sequences of all three ORFs display 29% identity, which increases to 71% when only YDR098c and YER174c are considered. The highest homology concentrates in two separate regions (Fig. 1A). The most N-terminal of these regions includes a common cysteine residue. In contrast with other glutaredoxins from yeasts or other prokaryotic or eukaryotic organisms (18, 29), a motif of two cysteine residues separated by two additional amino acids does not occur in the above three ORF products, although YPL059c contains a second cysteine in the most C-terminal homology region.

Comparisons were extended to a total of 23 putative glutaredoxin protein sequences present in the databases (Fig. 1B). This allowed us to define two subfamilies of glutaredoxins based on the sequence patterns of the two regions of highest homology, here referred to as regions N and C. Homology in region C (the most C-terminal) extends to all members of the two subfamilies, with a total of four residues conserved in all 23 proteins. In contrast, alignment in region N (the most N-terminal) was significant only when applied within each subfamily. Members of subfamily 1 all contain the motif PXCG/AFS/P (X being nondefined), with no other cysteine residue being present in this region, while subfamily 2 is defined by the above-mentioned motif CPY/FC. This motif partially defines the characterized active site of some glutaredoxins (43, 55), all of which are included in subfamily 2. No equivalent studies have been reported for subfamily 1 members. Interestingly, glutaredoxins of both subfamilies coexist in organisms ranging from bacteria (i.e., *E. coli*) to higher eukaryotes (such as humans) (Fig. 1B). In the case of *S. cerevisiae*, the previously characterized *GRX1*- and *GRX2*-encoded glutaredoxins (29) are ascribed to subfamily 2, while the products of YDR098c, YER174c, and YPL059w are subfamily 1 members. From the homology patterns and also from the determination of enzyme activities (see below), we propose to rename these last three ORFs *GRX3*, *GRX4*, and *GRX5*, respectively.

The Sequence Space approach (8) was used for a more detailed comparative analysis of the 23 glutaredoxin sequences, separately for regions N and C (Fig. 2). When this method was used to analyze region C in the whole set of sequences, the previously defined subfamily 1 clustered separately from the remaining sequences. Inside this cluster, Grx5 appears closer to glutaredoxins from multicellular eukaryotes than to yeast Grx3 and Grx4, which are positioned almost together. The 10 sequences of subfamily 2 were divided into three clusters corresponding respectively to bacterial molecules, mammalian molecules, and a cluster of yeast (*S. cerevisiae* Grx1 and Grx2 and *Schizosaccharomyces pombe*) and rice glutaredoxins. This same division in subfamily 2 glutaredoxins was confirmed from analysis of region N, with the difference that rice glutaredoxin mapped closer to *E. coli* glutaredoxins. Sequence analysis of region N in subfamily 1 confirmed the relative distance between Grx5 and Grx3/4. Although these three proteins are all positioned in the same cluster, the Grx5 sequence comes closer to *Arabidopsis thaliana* or human glutaredoxins than to Grx3 and Grx4.

A triple *grx3 grx4 grx5* mutant is not viable. In order to genetically characterize the new glutaredoxins, null individual mutants were obtained for each of the *GRX3*, *GRX4*, and *GRX5* loci and also double mutants derived from their respective crosses. Cultures of the *grx3* or *grx4* mutants displayed the same growth phenotype as the wild type both in rich and in SD-glucose minimal medium at the temperature intervals ranging from 15 to 37°C. In contrast, in *grx5* mutant cells growth rate was decreased by a factor of 1.6 compared to wild-type cells in YPD medium at 30°C (Table 2). Moreover, this mutant grew poorly in SD-glucose medium at 30°C and was unable to grow when the temperature was increased to 37°C. The *grx5* mutation was also linked to the inability to grow in YP-glycerol medium. Growth characteristics were even more affected in the *grx3 grx5* double mutant (although not in the *grx4 grx5* and *grx3 grx4* mutants) with respect to *grx5* mutant cells (Table 2).

In order to obtain the multiple mutant disrupted in all three glutaredoxin genes of subfamily 1, a *grx3 grx4* mutant strain was crossed with a *grx5* mutant. Fifty tetrads derived from the resulting diploid were analyzed, and yet no *grx3 grx4 grx5* multiple mutant could be isolated, in contrast to the other possible genotype combinations. To confirm that the above combination was not viable, we employed the *tetO* promoter substitution cassette (4) to substitute the chromosomal *GRX5* promoter for the doxycycline-regulatable *tetO* promoter in a *grx3 grx4* mutant background. The resulting strain was able to grow in the absence of doxycycline but arrested growth in the presence of the antibiotic (data not shown), confirming that inactivation of the three glutaredoxins was lethal for yeast cells.

Glutaredoxin-reduced activity causes protein oxidative damage in *grx3*, *grx4*, and *grx5* mutants under ordinary growth conditions. In order to prove that *GRX3*, *GRX4*, and *GRX5* code for proteins with glutaredoxin activity, we measured enzyme levels in the respective single mutants (Table 2). In *grx2* and *grx3* mutants, glutaredoxin activity decreased by 40% with respect to wild-type cells. It is remarkable that although *grx5* mutant cells showed growth defects not observed in the *grx3* or *grx4* mutants, the decrease in glutaredoxin levels in *grx5* mutant cells was only slightly higher than that in the other two mutants. While in the *grx3 grx4* double mutant there seemed to be a compensatory effect in activity levels relative to the respective single mutants, glutaredoxin activity in mutants affected in

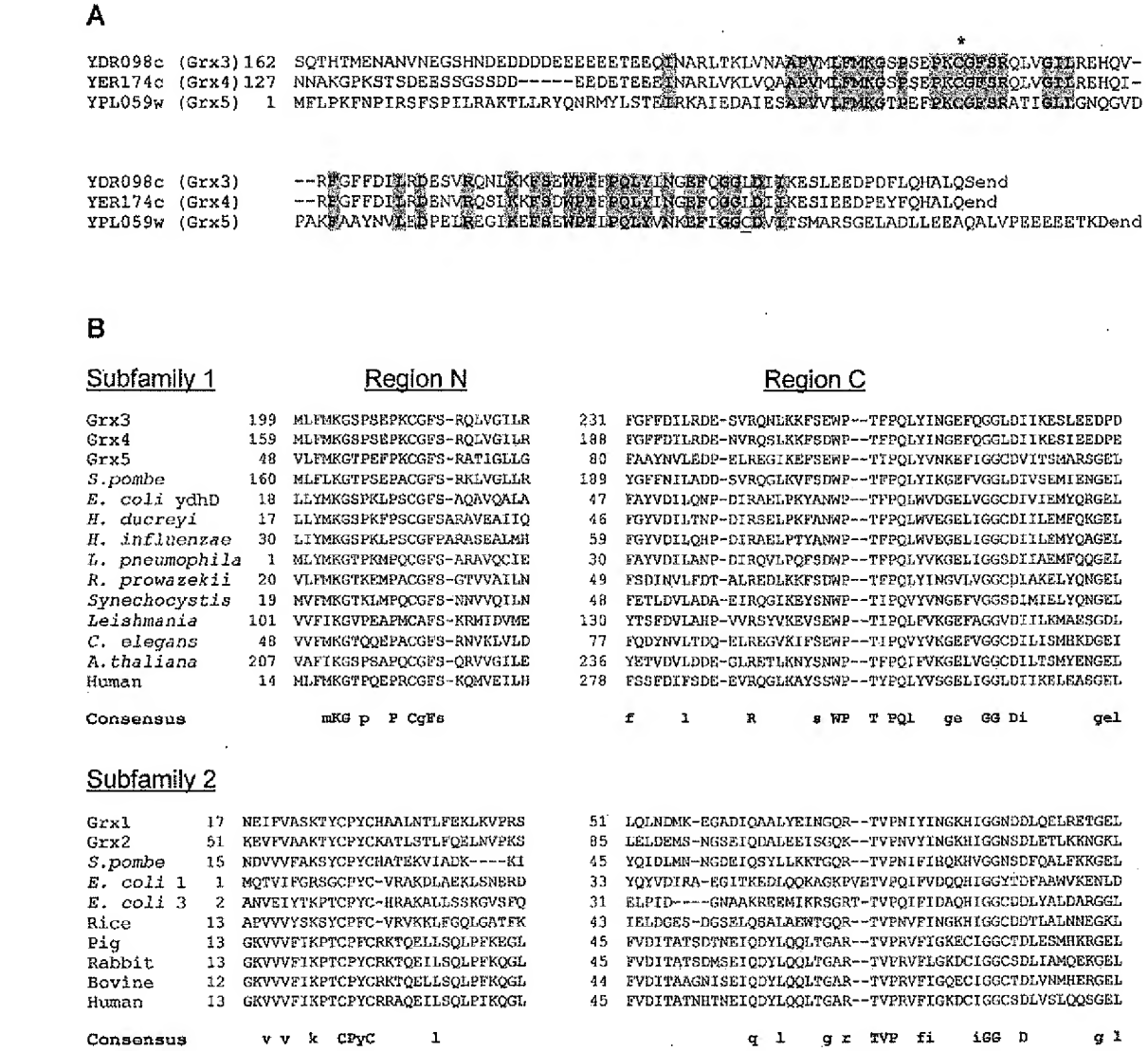

FIG. 1. Comparative analysis of glutaredoxin sequences. (A) Alignment of the *S. cerevisiae* Grx3, Grx4, and Grx5 amino acid sequences deduced from the nucleotide sequences of their respective ORFs. Common residues in the three sequences are shaded. The N-terminal extensions of Grx3 and Grx4 are not represented. The asterisk marks the common cysteine residue present in all three sequences. A second cysteine present in Grx5 is underlined. (B) Sequence analysis of relevant regions of 23 different glutaredoxin proteins. Regions N and C are respectively the most N- and C-terminal regions of the molecules for which significant alignments can be established. Sequences outside these two regions are not represented. Subfamilies 1 and 2 are initially defined according to the consensus sequences indicated in the figure. For the consensus sequences, residues identical in all members of each subfamily are represented in uppercase letters, while those common to at least 75% of them are in lowercase letters. More details about these sequences can be obtained from reference 36. *H. ducreyi*, *Haemophilus ducreyi*; *H. influenzae*, *Haemophilus influenzae*; *L. pneumophila*, *Legionella pneumophila*; *R. prowazekii*, *Rickettsia prowazekii*; *C. elegans*, *Caenorhabditis elegans*.

GRX5 plus one of the other two genes was similar to that in the single *grx5* mutant. Glutathione reductase activity, measured as a control, maintained equivalent levels in all the strains tested (Table 2).

It has been proposed that glutaredoxins participate in the maintenance of an adequate intracellular concentration of thiols, which play an antioxidant role in the cell (9, 34). Therefore, we tested whether the previously mentioned deletion mutants have higher basal levels of protein oxidative damage than wild-type cells. For this purpose, we measured the protein carbonyl content in crude extracts from cells grown in YPD medium. This parameter has been widely used to assess minimal values of protein damage under oxidative stress conditions (26, 48, 50). As shown in Table 2, single *grx3* and *grx4* mutants displayed a moderate increase in carbonyl content with respect to wild-type cells. This increase was more severe in the *grx5* mutant. In the case of the double mutants, the carbonyl content was slightly increased in *grx3 grx4* mutant cells and markedly increased in *grx5* mutant cells that also contained inactivating mutations in *GRX3* or *GRX4*. The effect of inactivation of the already-known glutaredoxin *GRX2* gene on protein oxidative damage was then checked in the same way (Table 2). In this case, the decreased levels of glutaredoxin enzymatic activity in the *grx2* mutant cells were also reflected in an increase in protein carbonyl content of about 15% with respect to wild-type cells. This was of the same magnitude as that in the *grx3* and *grx4* mutant cells but clearly less than the values obtained for the *grx5* mutant. In the case of the double mutants, a 50%

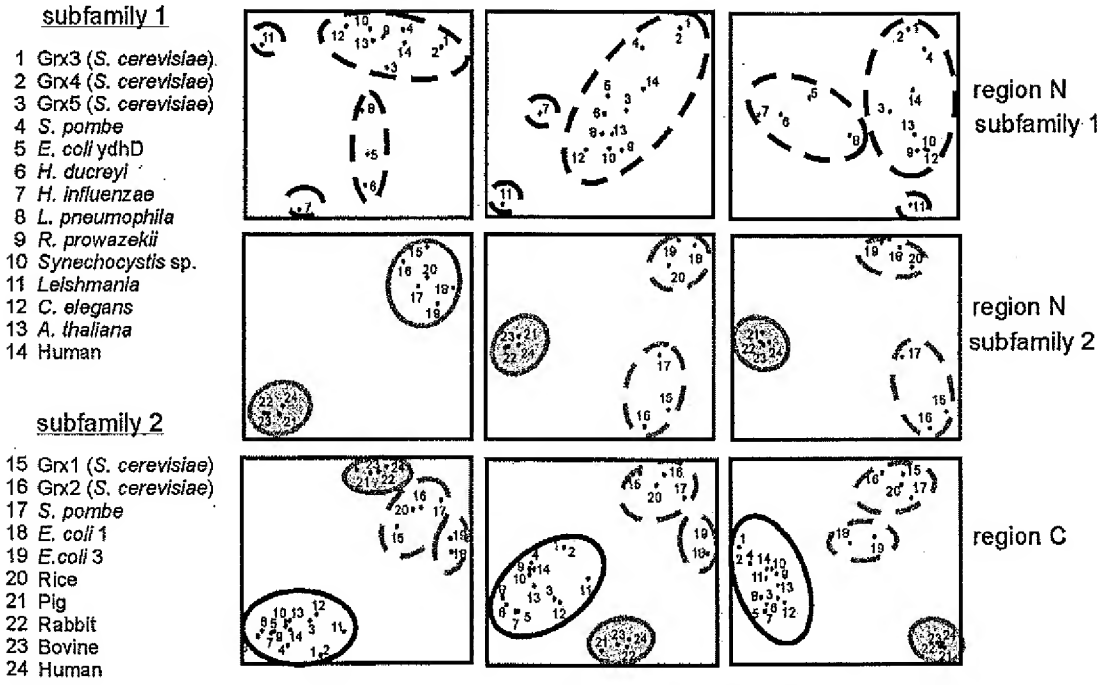

FIG. 2. Sequence Space analysis of the glutaredoxin family. Principal component analyses of the protein sequences are shown (from left to right) on the resulting 1-2, 1-3, and 2-3 discriminant axes (8). Analyses were carried out separately for region N in subfamily 1, region N in subfamily 2, and region C in the whole glutaredoxin family. Each point in the plots represents an individual sequence identified by a number. Distances between points are proportional to sequence divergence. Sequence clusters are defined according to proximity in the resulting plots (continuous lines). These clusters were tentatively divided into subsets of sequences (dashed lines) when the results on the three dimensions suggested the existence of relevant subgroups. See the Fig. 1 legend for genus abbreviations.

increase was observed for *grx2 grx3* mutant cells, and one of 30% was observed in the case of the *grx2 grx4* mutant.

To test whether the observed increases in protein carbonyl content in mutant cells affected the whole protein pool or only some proteins, we used Western blot analysis to compare the patterns of oxidized proteins exhibited by wild-type and mutant strains (Fig. 3). All the bands observed in wild-type cells that have to be considered background levels of protein oxidation increase in all mutants. Furthermore, in the mutants lacking *GRX5* at least one band appeared to be specifically oxidized (indicated by an asterisk in Fig. 3). This was not observed in the other mutant strains. By using crude extracts from *grx5* mutant cells, this protein band was purified to homogeneity by preparative electrophoresis, and its N terminus was sequenced. The protein was identified as transketolase. This was further confirmed by the N-terminal sequence of one oxidized peptide obtained after limited proteolysis of the whole protein with endoproteinase V8. Further extending these results, transketolase activity was measured in extracts from wild-type and *grx5* mutant cells. The latter exhibited only about 25% of the activity present in wild-type cells (20 versus 83 mU/mg of protein), confirming that oxidation leads to enzyme inactivation.

Grx5 glutaredoxin plays a central role in protection against induced oxidative and hyperosmotic stresses. Once it was

TABLE 2. Enzyme activities and protein oxidation levels in *grx* mutants[a]

Strain	Relevant genotype	Doubling time (min)[b]	Glutaredoxin activity[c]	Glutathione reductase activity[c]	Carbonyl content[d]		
					Control	Menadione	Hydrogen peroxide
CML235	Wild type	90 ± 4	51 ± 4	102 ± 10	0.69 ± 0.05	1.28 ± 0.05	0.96 ± 0.05
MML15	*grx3*	94 ± 1	30 ± 3	101 ± 6	0.78 ± 0.06	1.53 ± 0.05	1.10 ± 0.05
MML17	*grx4*	92 ± 5	28 ± 3	97 ± 6	0.75 ± 0.03	1.38 ± 0.05	1.08 ± 0.05
MML19	*grx5*	145 ± 11	22 ± 1	96 ± 6	0.99 ± 0.07	2.10 ± 0.05	1.63 ± 0.05
MML37	*grx3 grx5*	208 ± 9	19 ± 4	101 ± 10	1.16 ± 0.10	ND	ND
MML39	*grx4 grx5*	147 ± 3	21 ± 4	99 ± 9	1.15 ± 0.12	ND	ND
MML41	*grx3 grx4*	150 ± 16	44 ± 7	98 ± 12	0.89 ± 0.09	ND	ND
MML44	*grx2*	88 ± 2	14 ± 3	ND	0.81 ± 0.03	1.50 ± 0.10	0.95 ± 0.05
MML45	*grx2 grx3*	92 ± 1	19 ± 4	ND	0.89 ± 0.11	ND	ND
MML47	*grx2 grx4*	90 ± 2	16 ± 4	ND	1.09 ± 0.14	ND	ND
MML59	*grx2 grx3 grx4*	159 ± 7	8 ± 2	ND	1.25 ± 0.15	ND	ND

[a] Values are shown as means ± standard deviations. Three independent determinations were done in all cases. ND, not determined.
[b] In exponential cultures in YPD liquid medium at 30°C.
[c] Nanomoles of NADPH oxidized per minute per milligram of protein.
[d] Nanomoles of carbonyl groups per milligram of protein, in untreated cells (control) or cells treated for 1 h with 20 mM menadione or 2.5 mM hydrogen peroxide.

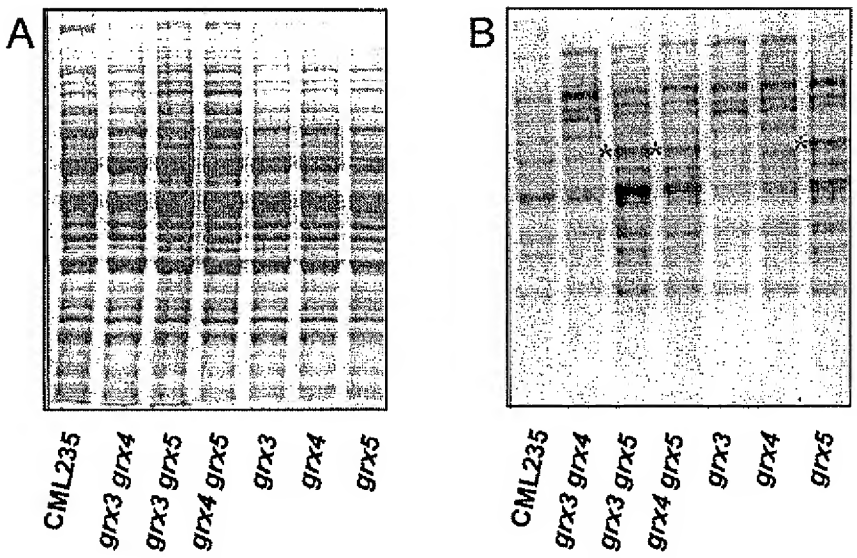

FIG. 3. Protein oxidative damage under normal growth conditions of wild type and *grx* single and double mutants. *MAT*a strains were employed. Cultures of wild type (CML235) and single and double mutants were grown in YPD liquid medium at 30°C until an optical density at 600 nm of 1 was reached. The crude extracts obtained were analyzed by Western blotting with anti-DNP antibodies (B). A parallel run stained with Coomassie brilliant blue is shown in panel A. Each lane contained 20 μg of total protein. Asterisks mark the identified transketolase band (see text for details).

demonstrated that the products of *GRX3*, *GRX4*, and *GRX5* are required for maintaining normal glutaredoxin levels in the cell, we next studied their role in protection against an externally induced oxidative stress. The effect of hydrogen peroxide and menadione (a generator of superoxide radicals) on viability was tested when they were applied to exponentially growing cells. Disruption of *GRX3* and *GRX4* had only a moderate effect on sensitivity to menadione and no effect on sensitivity to hydrogen peroxide, while disruption of *GRX5* caused a dramatic increase in sensitivity to both oxidants (Fig. 4A). The *grx3 grx5* and *grx4 grx5* double mutants were not markedly more sensitive to menadione and hydrogen peroxide than were *grx5* single mutants (Fig. 4B). In fact, survival after long-term treatment was slightly higher in *grx3 grx5* mutant cells than in *grx5* mutants, although this may be an effect of the lower growth rate of the double mutant.

Protein damage promoted by adding 20 mM menadione or 5 mM hydrogen peroxide to growing cells was analyzed by Western blotting (Fig. 4C). In these conditions, the *grx3* and *grx4* mutants revealed only a moderate increase in the level of protein oxidation with respect to wild-type cells, while a heavily oxidized protein band pattern was exhibited by the *grx5* mutant. For comparison, we included the already-described *grx2* mutant, which displayed a protein oxidation pattern similar to those observed in wild-type cells and *grx3* and *grx4* mutants. In agreement with the data presented in Table 2, the overall increase in carbonyl content in mutant cells was not due to a qualitative difference of oxidation in particular protein bands but to an increase in oxidative damage in most protein bands present in all the stressed strains.

Other authors have shown that some yeast mutants hypersensitive to oxidants are also more sensitive to osmotic stress (23). We therefore tested the sensitivity of *grx5* mutant cells to hypertonic conditions. This mutation increased sensitivity to high concentrations of KCl more than 10-fold, and the sensitivity was even higher in the double *grx3 grx5* mutant (Fig. 5A). To show that this effect was not caused by ion toxicity, we tested the effect of sorbitol at a concentration of 2 M or higher on transitory cell division arrest after the osmotic shock. In these conditions, growth was also more affected in *grx5* mutant cells than in the wild-type strain (Fig. 5B), confirming that the Grx5 product protects not only against oxidative stress but also against different types of hyperosmotic stress. On the other hand, none of the *grx3*, *grx4*, or *grx5* single mutants was more sensitive than wild-type cells to heat shock (shift from 25 to 37°C [data not shown]).

The hypersensitivity of *grx5* mutant cells to osmotic stress could have been caused by the effect of reactive oxygen species on cell wall architecture. To analyze this possibility, we tested the sensitivity of wild-type and *grx5* mutant cells to a number of especially toxic agents for cells altered in cell wall structure (31). *grx5* mutant cells did not show increased sensitivity (relative to wild-type cells) to calcofluor white, SDS, or caffeine (data not shown), thus eliminating the possibility of explaining increased osmotic sensitivity as being a direct consequence of hyperoxidation of cell wall molecules.

Grx2 and Grx5 functions can substitute for each other. Grx2 has been reported to account for most of the glutathione-dependent oxidoreductase activity of glutaredoxins in yeast cells and to play an important role in protection against hydrogen peroxide, but not against menadione (29). It was possible to obtain *grx2 grx3* and *grx2 grx4* mutant strains by standard genetic crosses from their respective single mutants, and they had significantly reduced oxidoreductase activity compared with single *grx3* or *grx4* mutants (see above and Table 2). However, no double *grx2 grx5* mutant could be obtained from a total of 40 tetrads analyzed. We conclude that this mutant combination is lethal and therefore that Grx2 activity can functionally substitute, at least in these particular conditions, for the loss of activity in *grx5* mutant cells. Loss of *GRX2* caused a less-than-threefold increase in sensitivity to hydrogen peroxide stress in cells grown in SD-glucose medium, and this was of the same order as that in the double *grx2 grx3* and *grx2 grx4* mutants (Fig. 6). Differences in sensitivity between wild-type and *grx2* mutant cells were even smaller in cultures grown in YPD medium (data not shown).

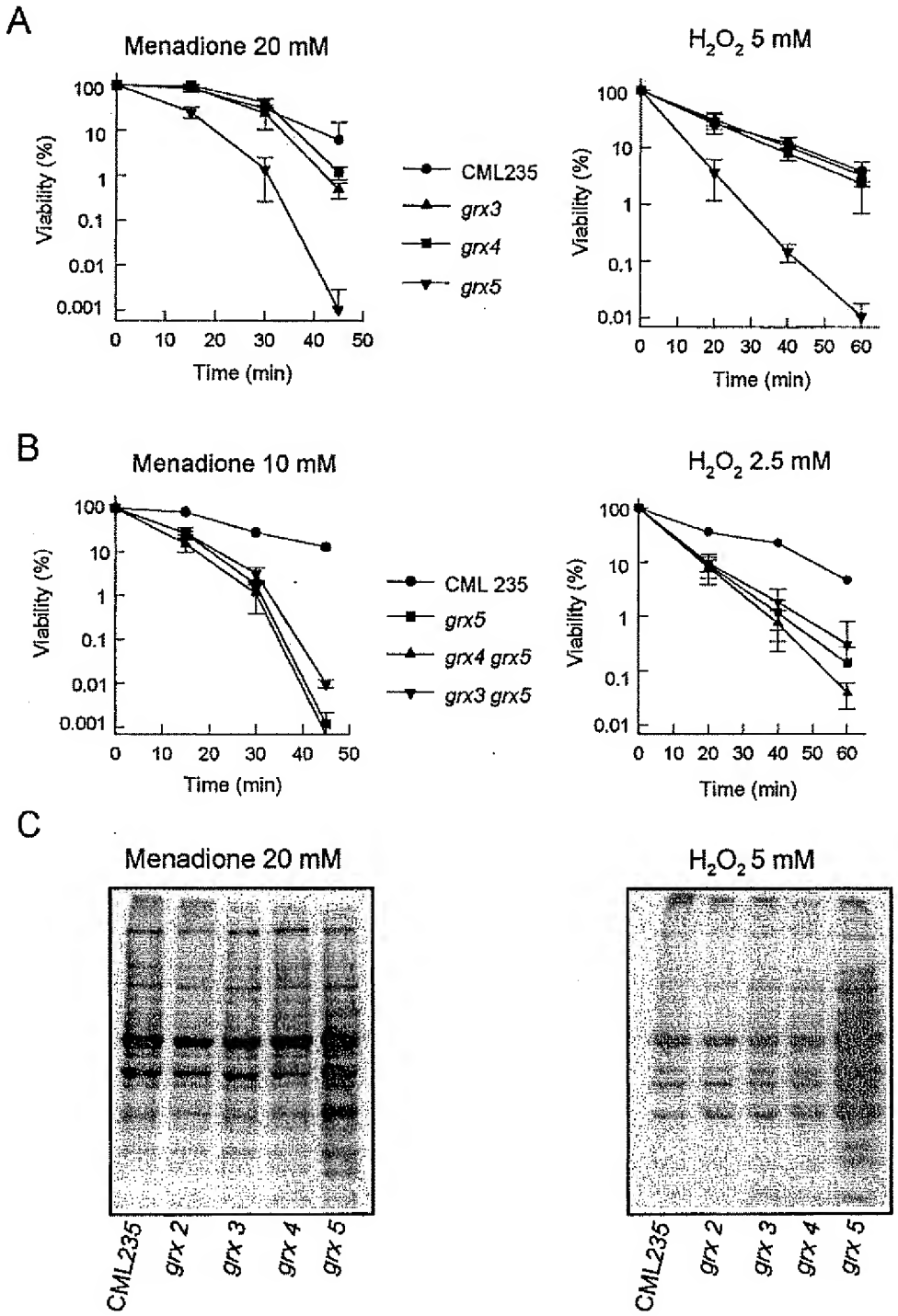

FIG. 4. Sensitivity of *S. cerevisiae grx* mutants to oxidative agents. *MAT*a strains were employed. (A) Cultures of wild-type (CML235) and single mutant strains growing exponentially in YPD liquid medium at 30°C were exposed to the indicated agents and concentrations, and viable numbers (relative to time zero values) were determined at different times. (B) As in panel A, except that lower agent concentrations were used to determine sensitivity of double mutants compared to wild-type and single mutant strains. (C) Protein oxidative damage in wild type and glutaredoxin mutants under stress conditions. Cultures of wild type and single glutaredoxin mutants were grown in YPD liquid medium at 30°C, and at an optical density at 600 nm of 1, menadione or hydrogen peroxide was added to the cultures at the final concentration of 20 or 5 mM, respectively. After 60 min of treatment, the cultures were harvested by centrifugation and crude extracts were obtained. Analyses by Western blotting with anti-DNP antibodies were conducted as described in Materials and Methods. Each lane contained 10 μg of total protein.

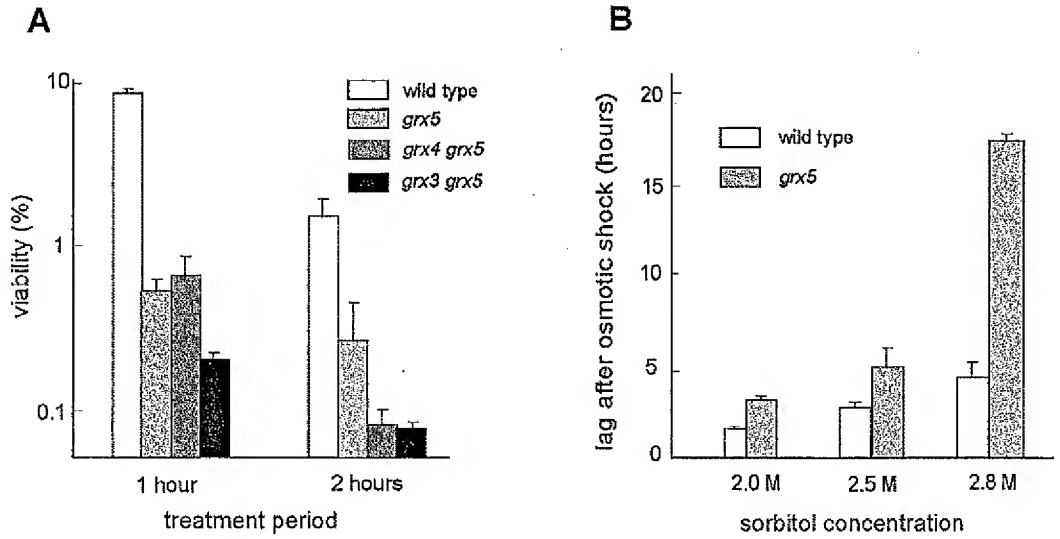

FIG. 5. Sensitivity of *S. cerevisiae grx* mutants to hyperosmotic treatments. (A) Exponentially growing wild-type (CML235) and mutant (*MAT*a type) cells in YPD medium at 30°C were supplemented with 2 M KCl, and cell viability (made relative to parallel untreated cultures) was determined at the indicated times. (B) Exponentially growing cells in YPD medium were treated with sorbitol at the final concentrations indicated, and incubation was continued under these conditions. Total cell numbers were measured at subsequent periods. Bars represent the lag periods after sorbitol addition during which cell division remained arrested before cultures resumed growth.

A triple *grx2 grx3 grx4* mutant was subsequently obtained by standard genetic crosses. Loss of the three genes caused about the same effect on cell growth rate in rich medium as the loss of the single *GRX5* gene (Table 2), although the multiple mutant grew more efficiently in minimal medium than did the *grx5* mutant (data not shown). Simultaneous disruption of *GRX2*, *GRX3*, and *GRX4* caused a 50% reduction in total cellular glutaredoxin activity compared to the single *grx2* mutant or the double *grx2 grx3* and *grx2 grx4* mutants. Correspondingly, total protein carbonylation was higher in the triple *grx2 grx3 grx4* mutant than in the other single and double mutants (Table 2), and sensitivity to hydrogen peroxide was higher in *grx2 grx3 grx4* mutant cells than in the single *grx2* mutant and of the same order as that in the *grx5* mutant (Fig. 6). We can conclude that although Grx2 and Grx5 can functionally substitute for each other, loss of Grx5 has more severe effects on cell physiology than loss of Grx2 alone and that in order to observe effects comparable to those of the loss of Grx5, it is necessary to simultaneously eliminate Grx2, Grx3, and Grx4.

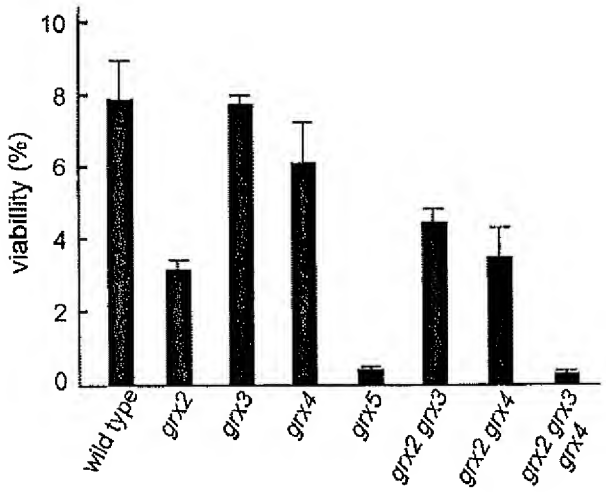

FIG. 6. Effect of oxidative stress (5 mM hydrogen peroxide for 1 h) on cell viability of *grx* mutants (*MAT*a strains) compared to that of wild-type cells (strain CML235). Cells were grown exponentially at 30°C in SD medium plus glucose, and after treatments, they were plated on YPD solid medium in order to determine viability. Bars indicate the percentages of viable cells relative to those in parallel untreated cultures.

Expression of the *GRX3-GRX4-GRX5* gene family in response to stresses. The transcriptional pattern of *GRX3*, *GRX4*, and *GRX5* was measured under several conditions (Fig. 7). Maximum expression for the three genes occurred during the exponential growth phase. As cells traversed the diauxic shift, transcript levels progressively decreased to under detectable levels in stationary phase. However, the rate of mRNA disappearance was different for each of the three genes. *GRX3* mRNA rapidly became undetectable, while *GRX4* expression was still detectable until the postdiauxic stage (Fig. 7). The expression of the three genes was not inducible under any of the three stresses applied (osmotic, oxidative with hydrogen peroxide or menadione, and heat). In fact, all three types of stress caused a reduction in the respective mRNA levels. This was moderate for *GRX5* and more intense for the other two transcripts. We can therefore conclude that the role of the Grx5 glutaredoxin in protection against oxidative and osmotic stresses does not depend on transcriptional changes induced by the respective type of stress.

DISCUSSION

Glutaredoxins are important for maintaining the reducing status of thiol groups in proteins (1, 9, 45). Together with thioredoxins, they are members of a superfamily of proteins that exert their activity through a disulfide exchange reaction involving one or two cysteine residues at the active site. The initially characterized members of the glutaredoxin family contained a CXXC active site, with XX being PY in most cases. Studies of phage T4 (43), pig (55), and *E. coli* (44) glutaredoxins have shown that of the two cysteine residues, only the most

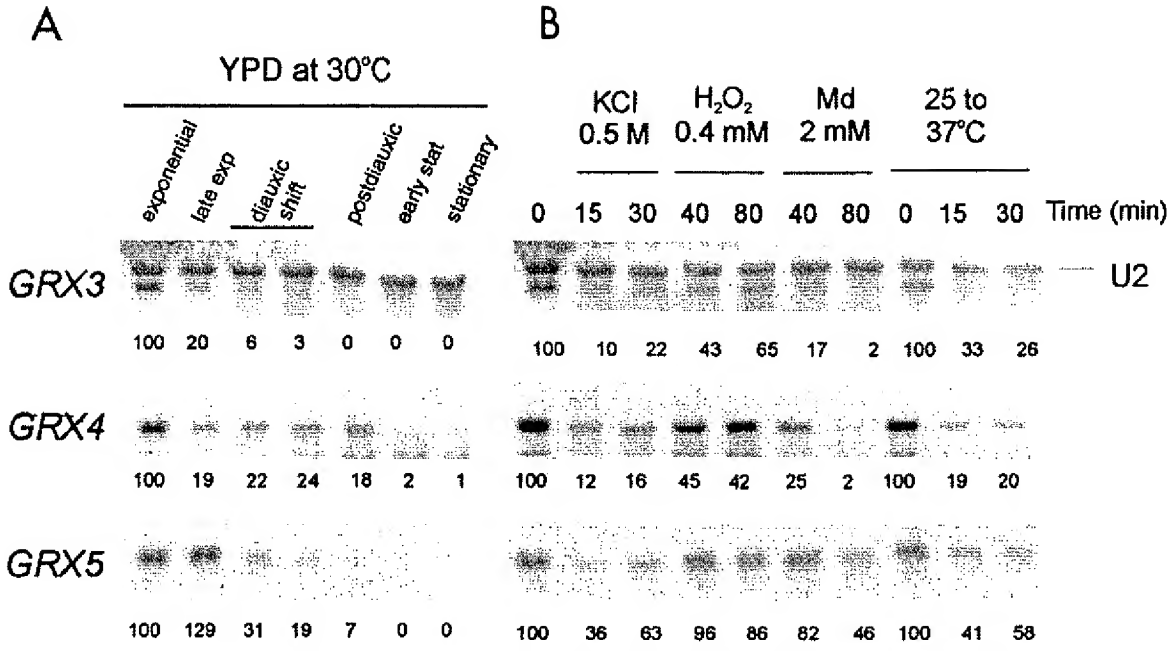

FIG. 7. Northern blot analyses of *GRX3*, *GRX4*, and *GRX5* expression. Samples were taken at different stages of the population growth curve in YPD liquid medium at 30°C (A) or after treatment of mid-exponential-phase cells (at 30°C except for heat shock) with KCl (0.5 M), hydrogen peroxide (0.4 mM), menadione (2 mM), or heat shock for the indicated times (B). Small nuclear U2 mRNA is shown as the loading control. Numbers under the lanes indicate the mRNA levels for each time point, relative to the mid-exponential-phase sample. For heat shock analysis, the time zero sample corresponds to exponential cultures at 25°C.

N-terminal is absolutely essential for enzyme activity, while mutants with mutations in the more C-terminal cysteine retain part of their GSH oxidoreductase activity (7, 43, 44, 55). Glutaredoxin-mediated protein glutathionylation has been explained in terms of the participation of a single cysteine (43). Studies involving an *E. coli* glutaredoxin mutated at the second cysteine residue indicate that both cysteine residues are required for reduction of protein disulfides (such as that in ribonucleotide reductase) through a dithiol mechanism, while the deglutathionylation of protein substrates would employ a monothiol mechanism (7) that could play an important physiological role at the endoplasmic reticulum for the maintenance of native protein conformation (30). Here we define a group of three new glutaredoxins (Grx3 to Grx5) in *S. cerevisiae* that structurally differ from the subfamily containing the CXXC motif at the active site and that constitute a separate subfamily including members ranging from bacterial to human glutaredoxins. Members of the latter subfamily contain the motif CG/AFS/P. The fact that this is the only conserved cysteine residue present in the members of this subfamily suggests that it may be part of the active site of the enzyme. Most members of this new subfamily also contain one or two basic amino acid residues separated by a few positions from the cysteine residue toward the C end. The presence of one or two basic residues close to the active site is also characteristic of the first subfamily, and it has been suggested that it might be needed for the thioltransferase reaction due to the enhancement of the S nucleophilicity of the reactive cysteine (55). This analogy reinforces the role of the CG/AFS/P motif in the reactivity of the glutaredoxins of the new subfamily.

The Grx3, Grx4, and Grx5 yeast glutaredoxins display sequence differences, though all three are members of the single-cysteine subfamily. Grx5 lacks part of an N-terminal domain present in Grx3 and Grx4. Application of the Sequence Space method (which allows sequence clustering based on amino acid conservation) has shown the Grx5 sequence to be closer to plant or mammalian glutaredoxin sequences than to Grx3 or Grx4. This method also permits us to observe that Grx1 and Grx2 yeast glutaredoxins are structurally separated from the Grx3/4/5 group. In the C-terminal region of homology, Grx5 contains the IGGC motif, which is absent in the other four yeast glutaredoxins but is present in the mammalian members of the cysteine-pair subfamily. The glycine pair in the above motif is common to all glutaredoxins of both subfamilies and might contribute to bringing an aspartic acid residue close to the active site cleft. The role of this conserved aspartic acid has been shown to be essential in the case of pig glutaredoxin (43).

Cell growth rate is not affected by single mutations in *GRX1* to *GRX4* (reference 29 and this work). In contrast, *grx5* mutant cells are constitutively affected in growth pattern (lower growth rate in rich medium, poor growth in minimal medium, and no growth in glycerol medium). Simultaneously, the *grx5* mutant has a higher basal protein carbonyl content than the other single glutaredoxin mutants. Since carbonyl content is employed as a measure of oxidative protein damage, the above observations could be interpreted as indicating that the Grx5 glutaredoxin has an important role in protection against oxidative damage of proteins during exponential growth. This could be correlated with the role of glutaredoxins in the homeostatic maintenance of intracellular thiols, which are necessary for several antioxidant activities in the cell (9, 34). In *E. coli*, protein oxidative damage is higher during respiratory growth conditions (50), and this also appears to be the case in *S. cerevisiae* (46), which would explain the inability of *grx5* cells to grow on glycerol when it is the only carbon source. The correlation cannot be extended, however, to all situations involving respiratory metabolism. Thus, during the postdiauxic growth stage, *GRX5* expression decreases and the viability of the *grx5* mutant is not affected. In this situation, yeast cells

perhaps employ alternative protection strategies against oxidative damage.

When cells are oxidatively stressed with menadione or hydrogen peroxide, the accumulation of protein damage is much higher in *grx5* mutant cells than in wild type or in the other *grx* mutant strains. This again correlates with the extreme effect of these situations on *grx5* mutant viability. Therefore, in those conditions in which an external oxidative stress is applied, there is a close relationship between the extent of protein carbonylation and the effect on cell growth, and these data confirm that Grx5 may be the most important glutaredoxin in protecting exponentially growing yeast cells against oxidative protein damage not only under normal growth conditions but also during induced stress. In carrying out this antioxidant function, Grx5 does not discriminate between the effects caused by menadione and those caused by hydrogen peroxide, in contrast with the protective role that Grx2 performs exclusively against hydrogen peroxide (29).

This relationship between protein carbonylation levels and growth defects has one exception. Simultaneous lack of Grx2, Grx3, and Grx4 has a more profound effect on constitutive protein oxidation than on cell growth. Also, the relationship cannot be strictly extrapolated to explain the relative contribution of each glutaredoxin species to overall cellular glutaredoxin activity. Total GSH oxidoreductase activity due to Grx5 alone seems to be similar to that of Grx3 or Grx4 but less than that of Grx2. However, the rate of growth is affected much more in *grx5* mutant cells. These differential effects of the *grx* mutations on growth could be explained by the fact that specific yeast glutaredoxins could identify individual protein substrates instead of acting as general GSH oxidoreductases. While inactivation of each *GRX3*, *GRX4*, or *GRX5* gene causes a general increase in oxidation levels of cell proteins, in the case of *grx5* mutant cells some individual protein bands (detected by Western blot immunoassay) are more prominently oxidized. Among these, transketolase, which is not detectable as an oxidized species in wild-type cells, appears to be particularly oxidized only in strains carrying the *grx5* mutation, even in a nonstressed situation. The finding that transketolase is especially susceptible to oxidative stress in yeast cells is relevant considering that it has been shown recently that *E. coli* transketolase activity is negatively affected in superoxide dismutase-deficient mutants, as well as in hyperoxia conditions (5). The presence of carbonyl groups in transketolase and the inactivation of the enzyme could both be a consequence of the highly oxidized environment created inside *grx5* mutant cells. Subsequently, since transketolase is involved in the pentose phosphate pathway, inactivation of this enzyme might lead to a depletion of NADPH levels, which would account for the lowered antioxidant capacity. Furthermore, this situation would block the possibility of redirecting carbohydrate metabolism to the regeneration of NADPH at the expense of glycolysis, which is what happens in wild-type cells a few minutes after hydrogen peroxide exposure (13). Under such circumstances, cell viability would obviously be compromised. Through depletion of erythrose-4-phosphate (which requires transketolase for its synthesis), superoxide dismutase deficiency causes auxotrophy for aromatic amino acids in *E. coli* (5). We tested whether the growth deficiency in *grx5* mutant cells in minimal medium was relieved by the addition of aromatic amino acids, but this was not the case (data not shown). Thus, although transketolase inactivation may contribute to growth deficiency in this particular situation, inactivation of other as-yet-uncharacterized proteins must be essential for the phenotype of Grx5-deficient cells.

Mutations in *GRX5* add to the list of oxidation-sensitive mutants which are also hypersensitive to osmotic stress (23). From our studies, the idea of a direct oxidative effect on cell wall architecture in *grx5* mutant cells should be discarded. Signal transduction pathways responding to hyperoxidative and hyperosmotic signals are interconnected in yeast. The pathway interrelationship is exemplified by Skn7, which is a signal transducer whose activity can be regulated by osmotic and oxidative stresses (6, 21, 27, 39). For the moment, no evidence to suggest that *GRX5* is a target for the pathways regulated by oxidative or osmotic signals exists, as the expression of *GRX5* is not induced by these stresses. Alternatively, the susceptibility of shared components of both types of pathways to the protein-hyperoxidation situation created in Grx5-deficient cells would result in sensitivity to oxidative and osmotic stresses.

The growth and stress sensitivity phenotypes of *grx* double mutants, together with the lethality of the *grx2 grx5* and *grx3 grx4 grx5* mutations, point to a central role of Grx5 in the regulation of the basal redox state of a number of functionally important proteins during exponential growth. Although we have not considered Grx1 glutaredoxin, as it has been shown to play only a minor role in exponential conditions (29), we have observed that a multiple *grx1 grx2 grx3 grx4* mutant is viable (our unpublished observations). These results could be explained by the existence of two different protein populations whose redox status could be separately regulated by the Grx1/2 and the Grx3/4 groups, respectively, while Grx5 would be able to act on both groups of protein substrates. Alternatively, the dithiol Grx1 and Grx2 enzymes and the monothiol Grx3, Grx4, and Grx5 enzymes could perform different thiol oxidoreductase activities, the first group reducing protein disulfides through a dithiol mechanism and the second group deglutathionylating glutathione-modified proteins through a monothiol mechanism (7, 30). Yeast cells would be unable to survive in the absence of the monothiol mechanism, but they would still be viable in the absence of the GSH-related dithiol one. In any case, Grx5 alone would be sufficient for maintaining the protein redox state, as it is able to replace the function of other glutaredoxins, at least when these are absent. This would also apply for an externally induced oxidative stress. In summary, Grx5 would act as a housekeeper for the adequate protein redox state during normal growth and as the agent responsible for the elimination of externally induced oxidative damage. Understanding the role of Grx5 and the other glutaredoxins will give us a better knowledge of how yeast cells protect themselves against constitutive and induced protein oxidative damage.

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Review

Plant glutaredoxins: still mysterious reducing systems

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Abstract. Glutaredoxins are ubiquitous oxidoreductases which are similar to thioredoxins and possess a typical glutathione-reducible CxxC or CxxS active site. We present here the current knowledge about these proteins in plants. At least 31 glutaredoxin genes are present in *Arabidopsis thaliana*, a value close to the thioredoxin gene number. Based essentially on active site sequences, a classification of these multiple genes is proposed. The specificity of the various apparently redundant forms within the glutaredoxin group or between glutaredoxin and thioredoxin can be analysed in terms of differential spatiotemporal expression of the genes, specificity vs. target proteins and mode of catalysis (glutathiolation/deglutathiolation processes appear to be a specific function of glutaredoxin). Additional putative functions are proposed for plant glutaredoxins based on their targets in other organisms and in the light of the existence of hybrid proteins containing glutaredoxin modules in their N- or C-terminal part.

Key words. Dithiol; glutaredoxin; glutathiolation; glutathione; monothiol; targets; thioredoxin.

Introduction

Glutaredoxins (Grx) are small ubiquitous oxidoreductases of the thioredoxin (Trx) family. The size of these proteins is generally ~10–15 kDa with an active site sequence CxxC or CxxS required for their redox properties [1]. Grx are maintained reduced with the help of NADPH, glutathione reductase (GR) and glutathione (GSH), whereas cytosolic and mitochondrial Trx are reduced by NADPH and NADPH thioredoxin reductase (NTR) [2–4]. In plants, various isoforms of Trx (Trx m, f, x, y and CDSP32 for chloroplast drought-induced protein of 32 kDa) are also present in the chloroplast [3, 5]. In this organelle, they are reduced via the electron transport chain with the help of two stromal proteins containing Fe-S clusters called ferredoxin and ferredoxin thioredoxin reductase [6]. GR and NTR belong to the pyridine nucleotide disulfide oxidoreductase family, which also comprises proteins such as lipoamide dehydrogenase, mercuric ion reductase and a bifunctional enzyme called Trx and GSSG reductase (TGR) [7]. These proteins are generally dimeric flavoproteins which possess a FAD binding domain, a NADPH binding domain and a dithiol/disulfide center of the CxxC or CxxxxC type. In most organisms, the Trx and GSH/Grx systems are the major reducing molecules and are thus involved in many cellular processes. Trx and Grx are multigenic families of proteins, represented by various isoforms. For example, in *Escherichia coli*, two bicysteinic Trx, three bicysteinic Grx have been characterized so far and one monocysteinic Grx (GenBank accession number NP_416171) also exists [8, 9]. In *Saccharomyces cerevisiae*, there are three bicysteinic Trx, including a mitochondrial isoform, two bicysteinic Grx and three monocysteinic Grx harbouring a CGFS active site [8, 9]. In mammals, there are two bicysteinic Trx or Grx isoforms, one cytosolic and one mitochondrial isoform of each protein and various Trx- or

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Grx-like proteins [8, 9]. The sequencing of complete or near complete genomes from *A. thaliana*, *Oryza sativa* or *Populus trichocarpa* and the occurrence of many expressed sequence tag (EST) sequencing projects for *Triticum aestivum*, *Zea mays*, *Chlamydomonas reinhardtii* and *Synechocystis* sp. indicate that a more complex diversity occurs in photosynthetic organisms. Indeed, up to 20–30 isoforms of Trx and Grx are present in some of these genomes, raising the question of the redundance and of the specificity of these isoforms, as Grx and Trx sometimes possess similar functions. Many review articles have described the various Trx systems and their functions in plant cells [3, 6]. This review will focus on plant Grx, for which information about expression, localization, and biochemical and structural properties are scarce.

The Grx content of the *A. thaliana* and *Populus* genomes has been analysed to propose a classification of plant Grx. Glutathiolation, one specific function of Grx, will be discussed in detail. The existence of many natural fusion proteins in plants or in other organisms between Grx modules and another module and some data about non-plant Grx targets will also be described in order to provide more information about other putative functions or target proteins of Grx.

The multigenic family of *grx* in genomes of photosynthetic organisms

The occurrence of nearly complete sequenced genomes or of EST-sequencing projects in plants provides valuable data about the abundance of Grx. We will describe below in detail the Grx content of two model plants, *A. thaliana*, an annual herbaceous species, and *P. trichocarpa*, a model of woody plants, and compare them briefly with the Grx content of other photosynthetic organisms.

A. thaliana

The analysis of the *Arabidopsis* genome in MATDB [MIPS (Munich information center for protein sequences) *Arabidopsis thaliana* database] (http://mips.gsf.de/proj/thal/db/index.html) indicates that at least 31 *grx* genes are present among the five nuclear chromosomes. Indeed, in higher plants, all the Grx are nuclear encoded but presumably exported in different compartments. Table 1 presents all the Grx found according to the number of conserved cysteines in the active site: there are 14 bicysteinic and 17 monocysteinic Grx. The MATDB protein entry codes, the size of the proteins including transit peptides, the putative localization and the sequence of the active sites, which is the hallmark of each Grx subclass, were also indicated for each Grx. All the localizations remain putative since no AtGrx (standing for *Arabidopsis thaliana* Grx, abbreviation also used later in the mansucript) has been characterized so far. Clearly, these data have to be taken cautiously, because recent studies indicate that many proteins could be exported into a subcellular compartment without any visible N- or C-terminal extension or could be targeted to several subcompartments. Nevertheless, most of the Grx are assumed to be cytosolic proteins. Among the bicysteinic proteins, three could be secreted, two localized in the chloroplasts but none is predicted to be mitochondrial. Among the monocysteinic proteins, four could be localized in chloroplasts and one in mitochondria, but none is predicted to be secreted.

Figures 1 and 2 present, respectively, an amino acid sequence comparison and a phylogenic tree of all AtGrx. Clearly, these analyses enable separation of the Grx into three classes, essentially as a function of the active site sequences. Only four amino acids are absolutely conserved among all AtGrx, the first cysteine of the active site, and proline, glycine and leucine residues located in the C-terminal part of the protein (fig. 1, in white on black). The identity between all AtGrx ranges from 5 to 95%.

The first well-defined class, characterized by a Cxx[C/S] or more precisely [Y/W]C[G/P/S]Y[C/S] active site, includes the four 'classical' dithiol Grx (CxxC1 to CxxC4) with CGYC, CPYC or CPFC active sites and two close isoforms (CxxC5 and CxxS12) with divergent WCSYC/S active sites. Most of the Grx characterized so far in other organisms belong to this group.

The second class includes four Grx with a CGFS active site (CxxS14 to CxxS17). CxxS17 is a fusion protein between a Trx motif (WASWCDAS active site) in the N-terminal part and three Grx motives (CGFS active site) in the C-terminal part. These proteins belong to the PICOT-HD (protein kinase C interacting cousin of Trx-homology domain) containing proteins [10]. The PICOT motif corresponds to a Grx module with a CGFS active site. This family thus includes, for example, the three monocysteinic Grx of *S. cerevisiae* (Grx 3–5) and one Grx of *Plasmodium falciparum* [11, 12].

The third class is the largest one and contains all the other Grx isoforms which possess an active site of the form CCx[C/S/G] or, more precisely, [S/T/G]CC[M/L][C/S/G]. Some of the proteins of the three classes contain additional cysteines likely to participate to the catalytic mechanism (see below).

Populus trichocarpa

The genome of *P. trichocarpa* is entirely sequenced (http://genome.jgi-psf.org/poplar0/poplar0.home.html) but not yet fully annotated. Nevertheless, large-scale EST sequencing provides more than 125,000 sequences. Up to now, 19 different Grx have been identified in the GenBank database by similarity search with AtGrx (table 1). Thus far, one major difference is the lack of many iso-

Table I. Glutaredoxin content of *A. thaliana* and *Populus sp.*

	Protein entry code	Length	Putative localization	Active site sequence	EST number from poplar
CxxC1	**At5g63030**	**125**	**cytosolic**	**YCGYC**	**BU867240**
CxxC2	**At5g40370**	**111**	**secretory pathway (P)**	**YCPYC**	**BU877060**
CxxC3	**At1g77370**	**130**	**secretory pathway**	**YCPYC**	**BU825153**
CxxC4	**At5g20500**	**135**	**secretory pathway**	**YCPYC**	**BU837457**
CxxC5	**At4g28730**	**174**	**plastidial**	**WCSYC**	**BU833604 ?**
CxxC6	At4g33040	144	cytosolic	SCCMC	BU883329
CxxC7	At3g02000	136	cytosolic (PM)	TCCMC	BU830321
CxxC8	At5g14070	140	cytosolic	TCCMC	?
CxxC9	At1g28480	137	cytosolic	GCCMC	BU811342
CxxC10	At5g11930	145	plastidial (C)	SCCMC	?
CxxC11	At3g62950	103	cytosolic (M)	SCCMC	BU811766
CxxC12	At2g47870	103	cytosolic (M)	SCCMC	BU889749
CxxC13	At2g47880	102	cytosolic	SCCLC	BU892497
CxxC14	At3g62960	102	cytosolic	SCCLC	CF230799
CxxS1	At1g03020	102	cytosolic	SCCMS	BU893638
CxxS2	At5g18600	102	cytosolic	SCCMS	?
CxxS3	At4g15700	102	cytosolic	SCCMS	BU895046
CxxS4	At4g15680	102	cytosolic	SCCMS	BU895046
CxxS5	At4g15690	102	cytosolic	SCCMS	BU895046
CxxS6	At3g62930	102	cytosolic	SCCMS	BU819383
CxxS7	At4g15670	102	cytosolic	SCCMS	BU895046
CxxS8	At4g15660	102	cytosolic	SCCMS	BU895046
CxxS9	At2g30540	102	cytosolic	SCCMS	?
CxxS10	At3g21460	102	mitochondrial	TCCMS	?
CxxS11	At1g06830	99	cytosolic	SCCLS	?
CxxS12	**At2g20270**	**179**	**plastidial (ER)**	**WCSYS**	**BU833604 ?**
CxxS13	At1g03850	150	plastidial (C)	GCCLG	?
CxxS14	*At3g54900*	*173*	*plastidial (M, ER)*	*MCGFS*	*BU875409*
CxxS15	*At3g15660*	*169*	*mitochondrial (P)*	*QCGFS*	*BU827149*
CxxS16	*At2g38270*	*293*	*plastidial (M)*	*QCGFS*	*BI132154*
CxxS17	*At4g04950*	*488*	*cytosolic*	*RCGFS, KCGFS (x2)*	*BI126366*

Data concerning *A. thaliana* Grx come from MATDB, and those of poplar Grx are EST sequences present in GenBank. Bold, normal and italic characters represents the three classes of Grx (CxxC/S, CCxC/S/G and CGFS active sites, respectively). Putative localizations are based on TargetP prediction software (http://www.cbs.dtu.dk/services/TargetP/). When other prediction softwares [Predotar (http://genoplante-info.infobiogen.fr/predotar/predotar.html)] and Psort (http://psort.nibb.ac.jp/form.html)) give different results, these are indicated between parentheses. Abbreviations: C, cytosol; ER, endoplasmic reticulum; M, mitochondria; P, plastid; PM, plasma membrane.

forms with a CCMS active site. The four classical Grx of the CxxC group and the four isoforms of the first group (CGFS) are present. There is also one isoform similar to AtCxxC5 or AtCxxS12, but we found only one EST, which is incomplete. Five CCMC and 2 CCLC different isoforms and 4 CCMS isoforms are also present in the database.

Other organisms

In *O. sativa*, *T. aestivum*, *Z. mays*, *Hordeum vulgare* and *Pinus taeda*, all classes are represented, but it seems there are not as many Grx with a CCxC/S active site in these organisms, whereas they are prominent in *A. thaliana*. It is likely that duplication events occurred in the *A. thaliana* genome for these isoforms. At present, it is not known whether these sequences are all expressed. For the organisms detailed below, the Grx isoforms present are constituted only by the expressed sequences, and since the full genome is not annotated, such duplication events are not yet detectable. In the green alga *C. reinhardtii* and in the cyanobacterium *Synechocystis PCC6803*, 6 and 3 Grx, respectively, are present either with a CxxC/S or a CGFS active site, but no isoforms with a CCxC/S active site [S. Lemaire, unpublished]. It is likely that the Grx of the CCxC/S group appeared later in the evolution and are specific for higher plants.

Catalytic and structural properties: is there a role for the second cysteine of the active site and for additional cysteines?

Very few biochemical and structural informations are available about plant Grx. Nevertheless, many structures of dithiol Grx from *Escherichia coli*, *Homo sapiens* or T_4 phage in oxidized and reduced forms have been resolved by nuclear magnetic resonance (NMR) spectroscopy or X-

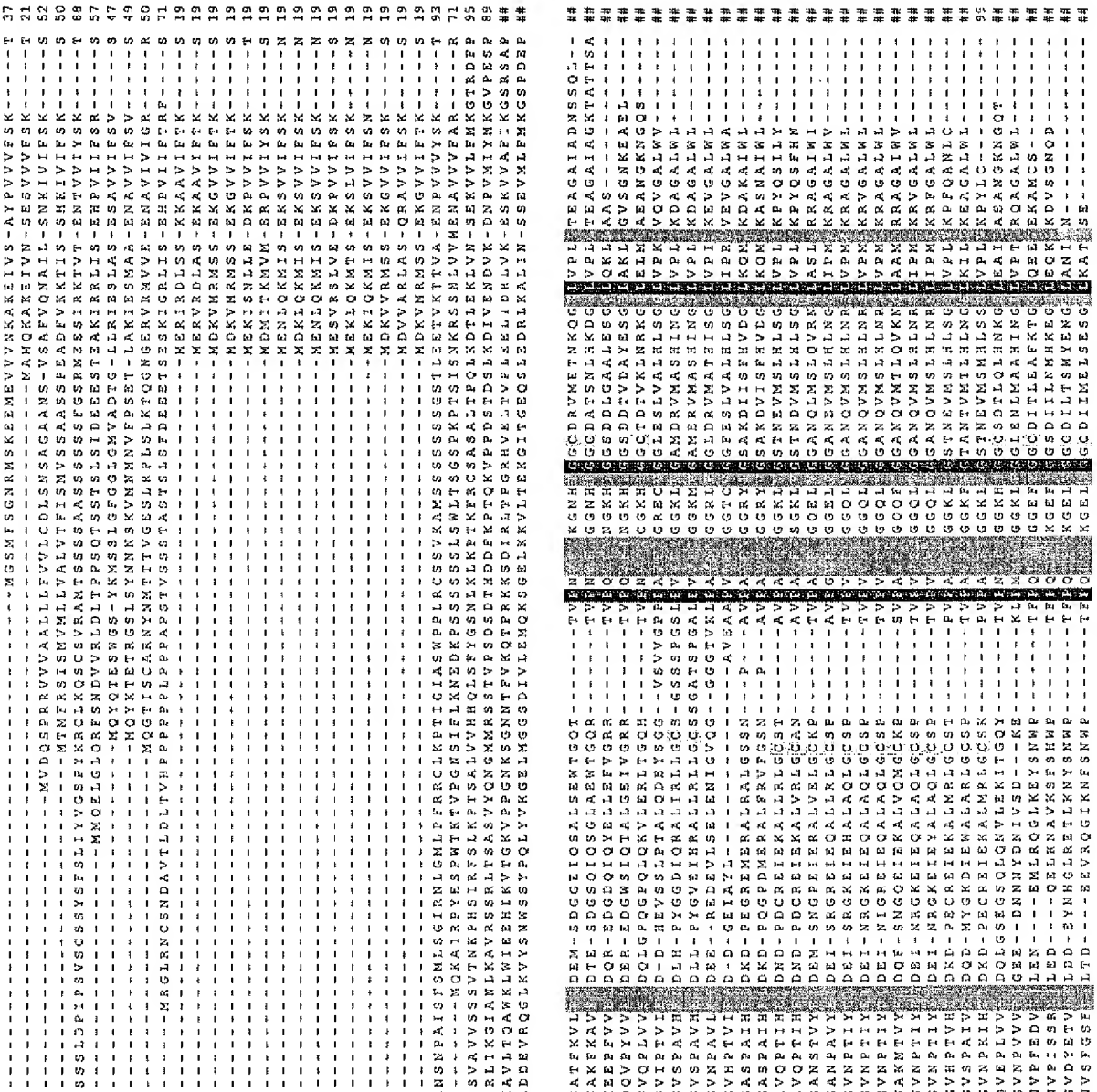

Figure 1. Amino acid sequence comparison of the 31 Grx of *A. thaliana*. The alignment was performed with ClustalW. The protein entry codes are similar to those of table 1. The strictly conserved amino acids are depicted in white on black; the conservative amino acid changes are indicated in white on gray. The second cysteine or serine of the active site and additional conserved cysteines are in black on gray.

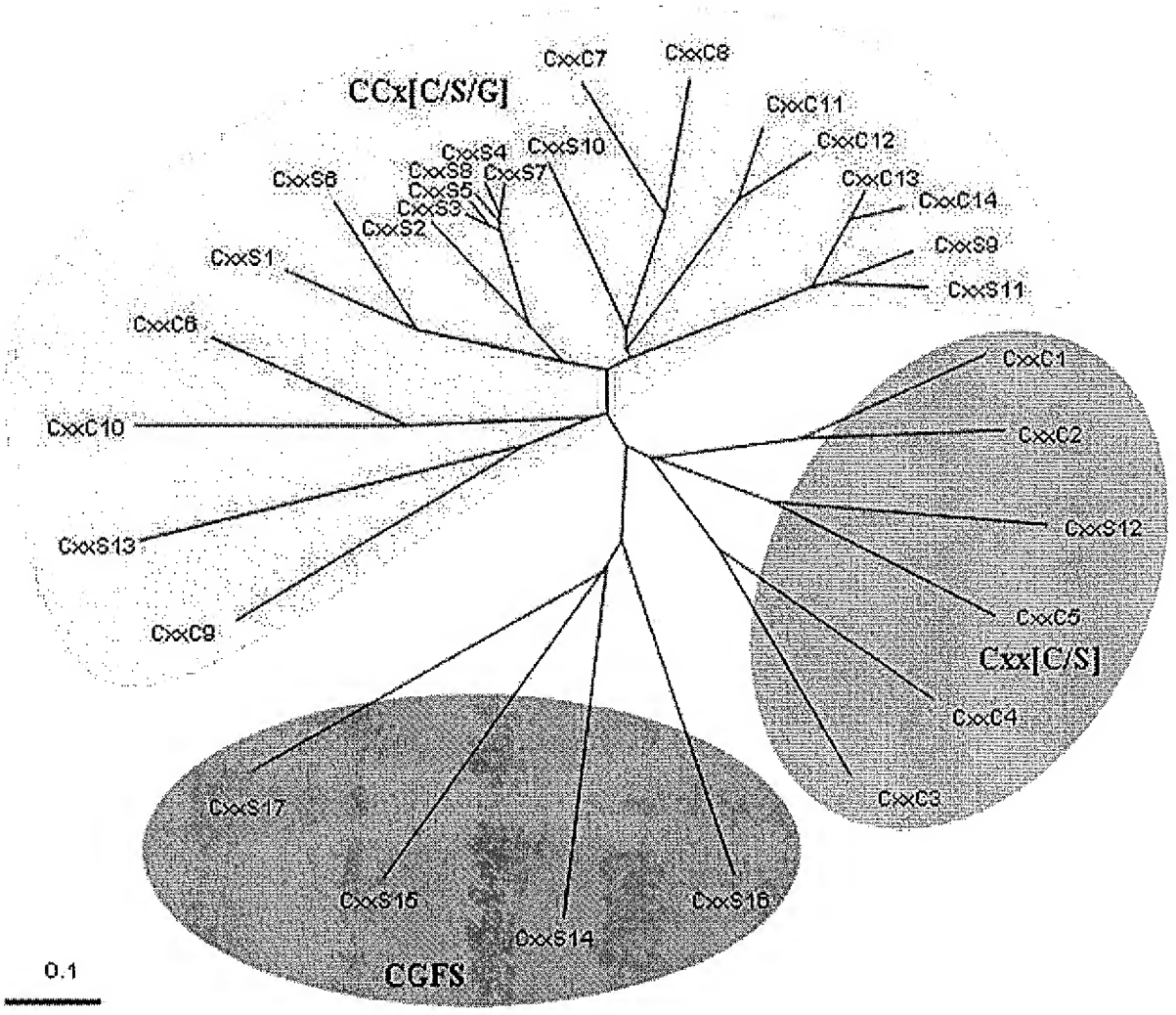

Figure 2. Phylogenetic tree of the various *A. thaliana* Grx isoforms. This tree was drawn using ClustalW. The protein entry codes are identical to those of table 1 and figure 1. Three classes can be distinguished according essentially to the active site sequences: CGFS, Cxx[C/S] and CCx[C/S/G].

ray crystallography (see [13] for a list). All the structures are organized into a Trx fold, consisting of a central β sheet surrounded by α helices. In terms of redox potential, Grx are considered to be weaker reductants than Trx, as their redox potentials are around –190 to –230 mV for *E. coli* Grx isoforms compared with –270 to –330 mV for Trx [14]. Only one poplar isoform with a classical YCPYC active site was characterized by site-directed mutagenesis in term of catalysis and structure [13, 15, 16]. The poplar Grx structure was resolved in complex with glutathione, and the most striking difference compared with the prokaryotic enzymes is the presence of an additional α helix in the N-terminus part [K. D'ambrosio et al., unpublished]. Biochemical studies demonstrate that only the first cysteine of the active site is essential for catalysis with dehydroascorbate (DHA) or type II peroxiredoxin (Prx), a peroxidase involved in the reduction of alkylhydroperoxides [15, 16]. Thus, classification into monocysteinic or bicysteinic Grx should be avoided, since recent data on the *S. cerevisiae* Grx 5 (CGFS active site) indicate that this protein possesses a disulfide bridge involving an extra active site cysteine [17]. This additional cysteine, found 50–54 amino acids after the active site in the consensus motif [I/V/F]G[G/A/S/T]C, is present in seven isoforms of AtGrx (see fig. 1), including CxxC/S Grx (AtGrx CxxC1, CxxC2, CxxC5 and CxxS12) and CGFS Grx (in AtGrx CxxS14, 16 and 17, but surprisingly not in AtGrx CxxS15). The role of this cysteine in the dithiol-containing Grx remains obscure. Its absence in one CGFS isoform and in the CCxC/S isoforms raises the question of the catalytic mechanism used by these isoforms. In the CCxC/S type, another cysteine is partially conserved in many isoforms in the consensus sequence [L/M]GC[S/K/A], located 37–38 amino acids after the active site (see fig. 1).

Expression and localization of glutaredoxins in plants

Very few data are available concerning the distribution of Grx in the different plant organs, and even less is known about their intracellular localizations. Initially, Grx has been identified in spinach leaves and then cloned from complementary DNA (cDNA) libraries of developing seeds of *O. sativa* or of cotyledons from *Ricinus communis* [18–20]. These two Grx, homologous to AtGrx CxxC2, present a distinct expression pattern. Whereas the rice *grx* is expressed exclusively in aleurone layers of seeds [19], the *grx* from *R. communis* is expressed not only in cotyledons but also in hypocotyls, in roots and to a lesser extent in leaves [20]. The gene encoding AtCxxS14 was shown to be expressed in leaves, stems and roots and very weakly in flowers, and it is repressed in seedlings by ion treatment [21]. Moreover, the Grx from *R. communis* is an abundant sieve tube protein of seedlings [20], and the poplar Grx, similar to AtGrx CxxC4, was also localized in the phloem sieve tubes by electronic microscopy and immunofluorescence [unpublished results].

Based on the abundance of each *grx* among the poplar EST in the GenBank database (search was stopped on 10/10/2003), we can estimate in silico the level of expression and the organ localization of each isoform. Out of a total of 114 ESTs encoding poplar Grx, 66 ESTs encode Grx with a CxxC/S active site, 28 encode Grx with a CCxC/S active site and 24 encode Grx with a CGFS active site. PtGrx CxxC2 and CxxC4 are the two most abundantly expressed isoforms (32 and 20 ESTs, respectively). CxxC2 is predominantly expressed in flowers, and CxxC4 in roots.

The functions of Grx in plants and in other organisms: known target proteins

Grx is able to reduce target proteins by dithiol-disulfide exchange using the two active site cysteines in a manner similar to Trx. On the other hand, Grx is a specific and efficient catalyst of protein-glutathione mixed disulfide reduction, a process called deglutathiolation [22]. For this mechanism, only the first cysteine of the active site is required. In animal cells and sometimes in bacteria or yeast, many proteins have been identified as being glutathiolated, especially in response to oxidative conditions and very often by using a proteomics approach. Table 2 presents a complete but non-exhaustive list of the plant and non-plant Grx targets, and indicates whether the target proteins are glutathiolated. If homologues are present in plants, all the targets found in other organisms are also potential interaction partners.

In plants, very little is known about the function of Grx. As their animal counterparts, some plant Grx isoforms are able to reduce dehydroascorbate into ascorbate [15, 20]. More interesting is the capacity of the plant Grx (CxxC type) to reduce the type II Prx [23, 24]. On the other hand, one Grx (CxxC/S type) of *O. sativa* was found to exhibit a GSH-dependent peroxidase activity toward various hydroperoxides as described for the two bicysteinic Grx of *S. cerevisiae* [25, 26]. This is quite surprising since *O. sativa* possesses at least one type II Prx isoform (EST accession number BP184892). The poplar Grx characterized, which belongs to the same group of Grx, does not possess such an activity toward hydroperoxides [27]. Other known targets of Grx in *A. thaliana* are H^+/Ca^{2+} transporters called CAX1 and CAX4 for cation exchanger [21]. Both AtGrx CxxS14 and 16 exhibiting a CGFS active site were found to activate these transporters, probably by a direct interaction which could disrupt the autoinhibition of these transporters. Finally, two proteins of the sugar metabolism from *A. thaliana*, an aldolase and a triose phosphate isomerase, have been found to be glutathiolated and are thus potential targets of Grx for deglutathiolation [28].

In organisms other than plants, Grx are already known to be involved in many processes, such as apoptosis [29], iron sulfur assembly in mitochondria [30, 31] and virion morphogenesis [32]. Moreover, Grx seem to be very important in transduction signaling pathways, as they regulate many transcription factors, kinases and phosphatases; in stress response by regulating various antioxidative enzymes such as Prx and GSH peroxidase; and in cytoskeleton organization, as many proteins are glutathiolated (table 2).

Moreover, many enzymes, listed in table 2, involved in various metabolic pathways are regulated, either by dithiol disulfide exchange or by glutathiolation. One interesting example is the *E. coli* PAPS (3′-phosphoadenylylsulfate) reductase. This enzyme possesses one cysteinyl residue per subunit. In the oxidized form, the enzyme consists of two subunits with an intermolecular disulfide bond, which could be reduced either by Grx or Trx [33]. On the other hand, this cysteine can also be glutathiolated. In this case, only Grx is able to remove GSH [34].

Finally, data on plant target proteins of Grx are scarce compared with other organisms, whereas the number of genes encoding Grx suggests a high representation of this kind of protein. The development of proteomics tools and the emergence of more complete protein databases should allow the identification of new targets. One way, similar to that used for Trx, could be to construct affinity columns with monocysteinic Grx and to retain selectively some covalently interacting proteins, or to use specific probes of thiol groups such as monobromobimane [35, 36]. Another way is to identify glutathiolated proteins by following the methods used for the animal cells, i.e. (i) detection using radiolabeled or biotinylated GSH [28, 37] or (ii) using alkylating agents such as iodoacetamide or N-ethylmaleimide biotin [38, 39].

Table 2. Glutaredoxin target proteins.

	References
Plant Grx targets	
Poplar type II Prx	[23]
Arabidopsis H^+/Ca^{2+} transporter	[21]
Arabidopsis triosephosphate isomerase*	[28]
Arabidopsis aldolase*	[28]
Nonplant Grx targets	
Kinases/phosphatases/transcription factors/signal transduction pathways	
E. coli OxyR	[50]
Human NF1* (nuclear factor 1)	[51]
Rat protein kinase C	[52]
Mouse PEBP2 (polyoma enhancer binding proteins 2)	[53]
Human PTP1B* (protein tyrosine phosphatase 1B)	[54]
Rat H-Ras*	[39]
Human NF-κB (nuclear factor κB) (p50 subunit)	[55, 56]
Human AP1 (activator protein 1)* (c-jun subunit)	[55, 56]
Human CREB (cyclic AMP-response element binding protein)	[55]
Human caspase-3*	[56]
Human Ref1 (redox factor 1)	[57]
Human ASK1 (apoptosis signal-regulating kinase 1)	[58]
Human CRK-like protein*	[38]
Human protein phosphatase 2A	[59]
Mouse cAMP-dependent protein kinase	[60]
Human Akt (Ser/Thr kinase)	[61]
Human Ran-specific GTPase activating protein*	[37]
DNA, RNA, protein synthesis, folding and degradation	
E. coli ribonucleotide reductase	[2]
HIV protease*	[62]
Human cathepsin K*	[63]
Human ubiquitin-conjugating enzymes*	[64, 65]
Human endoplasmic reticulum protein*	[64]
Human SFR1 splicing factor*	[64]
Human 40S ribosomal protein S12*	[64]
Human heat shock cognate 71-kDa protein*	[38]
Human HSP70*	[37,64]
Human HSP60*	[38,64]
Human heat shock protein HSP 90-β*	[38]
Human cyclophilin A*	[37,64]
Human protein disulfide isomerase*	[38,64]
Human translation initiation factor 6*	[38]
Human translation elongation factor*	[64]
Human 40S ribosomal protein SA*	[38]
Human prolyl 4-hydroxylase alpha subunit*	[38]
Human RNA binding protein regulatory subunit*	[64]
Human 14-3-3 protein*	[38]
Human aspartyl-tRNA synthetase*	[38]
Human endoplasmin*	[38]
Human ubiquitin*	[37]
Rat heat shock cognate 70-kDa fragment*	[37]
Saccharomyces cerevisiae 20S proteasome	[66]
Cytoskeleton	
Human actin*	[38, 64, 67]
Human tubulin β1*	[38]
Human vimentin*	[64]
Human laminin*	[38]
Human tropomyosin*	[38, 64]
Human transgelin*	[64]
Human cofilin*	[64]
Human myosin*	[37, 64]
Human profilin*	[37, 64]
Stress response/redox regulation	
Human glutathione peroxidase	[68]
Bovine Cu,Zn superoxide dismutase*	[56]
Human metalloproteinases	[69]

Table 2 (continued)

	References
Human Trx*	[48]
Human peroxiredoxin 1*	[38, 64]
Human peroxiredoxin 4*	[38]
Human peroxiredoxin 6*	[38]
Human stress-induced phosphoprotein 1*	[64]
Rat peroxiredoxin 5*	[37]
Metabolism/energetics	
Rat or human pyruvate kinase*	[38, 70]
Rat ornithine decarboxylase	[71]
Human phosphofructokinase	[72]
Rat S-adenosylmethionine synthetase	[73]
Human aldose reductase*	[38, 74]
Human glyceraldehyde 3-phosphate dehydrogenase*	[75]
E. coli PAPS reductase (*)	[33, 34]
E. coli arsenate reductase*	[76]
Rabbit Ca^{2+} ATPase*	[77]
Rabbit glycogen phosphorylase b*	[39]
Rabbit glycerol phosphate dehydrogenase*	[56]
Bovine haemoglobin*	[56]
Rabbit creatine kinase*	[56]
Yeast alcohol dehydrogenase*	[56]
Rat malate dehydrogenase*	[78]
Human inosine 5′-monophosphate dehydrogenase 2*	[38, 64]
Human enolase*	[38, 64]
Human phosphoglycerate kinase*	[64]
Human, aldolase*	[64]
Human 6-phosphogluconolactonase*	[37, 64]
Human phosphorylase kinase δ*	[64]
Human, triosephosphate isomerase*	[64]
Human dUTP pyrophosphatase*	[64]
Human and rat cytochrome c oxidase*	[37, 64]
Human fructose bisphosphate aldolase A*	[38]
Human nicotinamide N-methyltransferase*	[38]
Human inorganic pyrophosphatase*	[38]
Human fatty acid-binding protein*	[64]
Human β-galactoside soluble protein*	[64]
Human 3-hydroacyl-CoA dehydrogenase type II*	[38]
Human glucose-regulated protein*	[38]
Human histamine release factor*	[38]
Human L-lactate dehydrogenase*	[38]
Human zyrosine hydroxylase	[79]
Human glucosidase II*	[38]
Rat mitochondrial complex I (51- and 75-kDa subunits)	[80]
Rat α-ketoglutarate dehydrogenase*	[81]
Rat enoyl CoA hydratase*	[37]
Other functions	
Human carbonic anhydrase III*	[39, 82]
Human annexin II	[83]
Bovine serum albumin*	[56]
Rat neurogranin/RC3	[84]
Rat neuromodulin/GAP-43	[84]
Human nudix-type motif 6*	[64]
Human T complex protein 1*	[64]
Human lymphocyte-specific protein 1*	[64]
Human nucleosidediphosphate kinase A*	[38]
Human hepatoma-derived growth factor*	[38, 64]
Human ash protein*	[64]
Human My032 protein*	[64]
Human nucleophosmin*	[37, 64]
Human histidine triad nucleotide-binding protein 2*	[37]

Proteins identified as glutathiolated are labelled with an asterisk (*). In other cases, they interact either via dithiol-disulfide exchange, or the mode of catalysis is unknown.

Fusion proteins with a Grx module: physiological significance

In prokaryotes, the genes coding for two proteins which interact with one another are often associated in the same gene cluster or fused together. Computational methods based on phylogenetic profiles [40] and on genome analyses were developed to detect fusion proteins [41] or gene clusters [42]. This organization suggests a possible redox interaction between these proteins in organisms where they are not fused. An example concerning redox-regulated proteins is the fusion between Trx and Trx reductase modules in some bacteria such as *Mycobacterium leprae* [43] or in *A. thaliana* (MATDB protein entry code At2g41680). In all other organisms, these proteins, which also need to interact, are produced as separate proteins.

Using CDART (Conserved Domain Architecture Retrieval Tool, http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps), various motives can be detected in proteins [44]. These motifs are classified by COG (clusters of orthologous groups of proteins) or pfam (protein families and HMMs) entry codes. In order to identify putative new targets of Grx, this program was used to detect hybrid proteins containing one or many Grx modules (COG0695). Figure 3 presents the schematic organization of these hybrid proteins.

In plants, this type of fusion proteins was already described. The APS (5′-adenylylsulfate) reductase, an enzyme involved in the sulfur metabolism, is coupled to a Trx motif in the C-terminus which possesses a Grx activity [45]. In the red alga *Gracilaria gracilis*, a functional protein is constituted by two Grx modules fused in the N-terminal part to a methionine sulfoxide reductase of type A (MsrA) (COG0225) [N. Rouhier et al., unpublished]. Up to now, only Trx was demonstrated able to reduce MsrA. The existence of this hybrid protein strongly suggests that some MsrA isoforms could be reduced by Grx.

Another example of fusion proteins are enzymes made of a Prx (COG0678) coupled to a Grx domain in the C-terminus. This type of protein, found essentially in bacteria, is functional in hydroperoxide reduction in the presence of GSH as a donor [46]. In all other organisms, the two proteins are not fused. Interestingly, in higher plants the two enzymes, produced as distinct proteins, can interact [23, 24].

An interesting protein, found in eukaryotic organisms such as mammals or platyhelminthes, is constituted by a Grx domain in the N-terminus fused to a Trx reductase (COG1249) in the C-terminus [7]. This protein is 'trifunctional', as it possesses Trx reductase, GR and Grx activities. The previously described PICOT-HD features proteins comprising a Trx-like motif (pfam00085) in the N-terminus associated with one, two and even three Grx modules in the case of AtGrx CxxS17 (fig. 3).

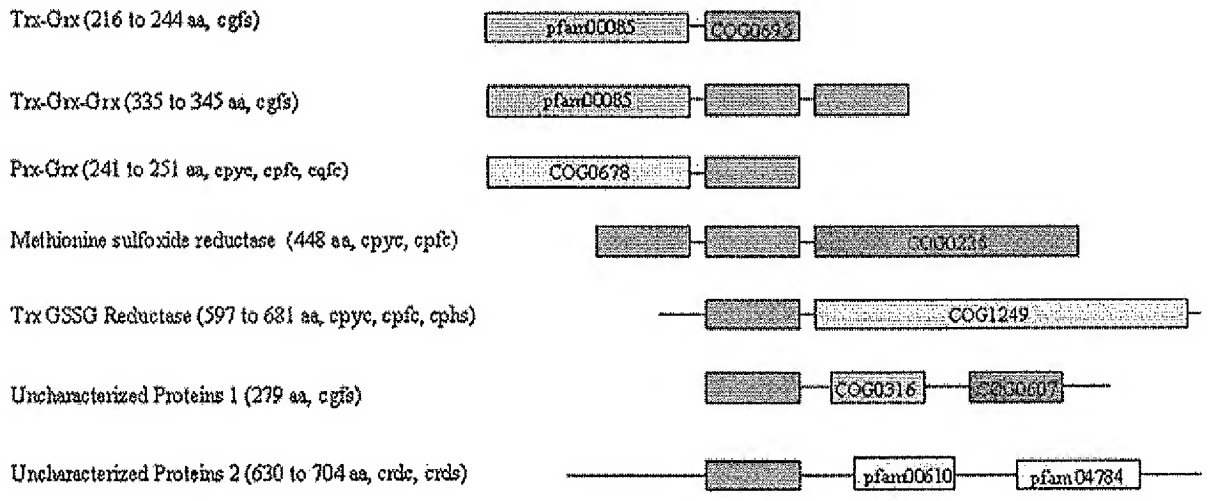

Figure 3. Fusion proteins containing one or many Grx modules. The hybrid proteins were found using the CDART program available at http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi. The pfam or COG entry codes of the different colored domains are indicated. The size of the proteins in amino acids and the sequences of the Grx active site are indicated between parentheses. The accession numbers characteristic for each type of protein are as follows. Trx-Grx XP_311699, *Anopheles gambiae*; AA053174, *Dictyostelium discoideum*; NP_609641, *Drosophila melanogaster*; NP_596647, *Schizosaccharomyces pombe*. Trx-Grx-Grx: NP_741524, *Caenorhabditis elegans*; NP_075629, *Mus musculus*; AAF28844, *Homo sapiens*. Prx-Grx: NP_485581, *Nostoc sp*; NP_407361, *Yersinia pestis*; NP_246286, *Pasteurella multocida*; NP_273984, *Neisseria meningitidis*; NP_232265, *Vibrio cholerae*. Methionine sulfoxide reductase: AF121271, *Gracilaria gracilis*. TGR: AAN63052, *Echinococcus granulosus*; AAK85233, *Schistosoma mansoni*; NP_694802, *Mus musculus*; AAH50032, *Homo sapiens*. Uncharacterized protein 1: NP_638714, *Xanthomonas campestris*; NP_299673, *Xylella fastidiosa*. Uncharacterized protein 2: AAM91894, *Oryza sativa*; P_566405, *A. thaliana*.

Other proteins, uncharacterized so far, contain one Grx module associated with various protein motifs of unknown function. First, some proteins, found in *Xylella fastidiosa* and *Xanthomonas campestris* or *axonopodis* and consisting of 279 amino acids, possess a Grx module followed by a domain of unknown function called IscA (COG0316) and a domain called PspE (COG0607) related to rhodanese sulfurtransferase. Other plant proteins (from *O. sativa* and *A. thaliana*), of larger size (from 630 to 700 amino acids), present a similar architecture. It consists of a Grx domain followed by a domain of unknown function (DEP, pfam00610) found in various signaling proteins such as Dishevelled, Egl-10 or Pleckstrin, and another conserved domain of unknown function (DUF547, pfam04784).

Crosstalk between GSH/Grx and Trx systems

The analysis of *S. cerevisiae* mutant strains for Trx reductase, GR and for the bicysteinic Trx or Grx suggests that the redox state of the Trx system is maintained independent of the GSH/Grx system [47]. Nevertheless, some data indicate that the two systems are dependent on one another. First, the yeast mitochondrial monocysteinic Grx5 is efficiently reduced by *E. coli* Trx compared with GSH [17]. The reverse example is the reduction of a poplar Trx by a poplar Grx or by *E. coli* Grx1, 2 or 3, but not by NTR [85]. Moreover, a cysteine residue in position 72 of human Trx, which does not belong to the active site, is glutathiolated in response to an oxidant, this modification abolishing its activity [48]. It is likely that Grx could regulate the Trx system by the deglutathiolation process. Another example of the complexity of these systems is the GSSG reduction by the Trx system in some organisms such as *D. melanogaster* which lack GR [49]. Finally, as mentioned above, the TGR protein is another example of the interconnection between the two systems because it is able to reduce both Trx and GSSG and to use GSH as a donor for the Grx module [7].

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Exploring the active site of plant glutaredoxin by site-directed mutagenesis

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Abstract Six mutants (Y26A, C27S, Y29F, Y29P, C30S and Y26W/Y29P) have been engineered in order to explore the active site of poplar glutaredoxin (Grx) (Y_{26}CPYC_{30}). The cysteinic mutants indicate that Cys 27 is the primary nucleophile. Phe is a good substitute for Tyr 29, but the Y29P mutant was inactive. The Y26A mutation caused a moderate loss of activity. The YCPPC and WCPPC mutations did not improve the reactivity of Grx with the chloroplastic NADP-malate dehydrogenase, a well known target of thioredoxins (Trxs). The results are discussed in relation with the known biochemical properties of Grx and Trx.

Key words: Glutaredoxin; Thioredoxin; Redox regulation; Dithiol/disulfide exchange

1. Introduction

Thioredoxin (Trx) and glutaredoxin (Grx) are structurally related proteins, the major function of which is to reduce disulfide bridges on other proteins. Both proteins differ by their mode of reduction and their active site sequence (YCP[Y/F]C for Grx and WC[G/P]PC for Trx) [1].

Trxs are fairly well characterized in plants, and it has been shown that there are multiple nuclear genes (close to 20 in the simple model *Arabidopsis thaliana* that encode cytosolic, mitochondrial and chloroplastic isoforms) [2]. In the cytosol and probably mitochondria, Trxs are reduced via NADPH and a flavoprotein, NADPH Trx reductase. Chloroplastic Trxs are reduced in the light by a cascade that involves the photosystem I, [2Fe2S] stromal ferredoxin and an iron–sulfur enzyme, ferredoxin–Trx reductase [3]. The targets of chloroplastic Trxs are well characterized, notably the redox-regulated fructose-1,6-bisphosphatase and NADP-malate dehydrogenase (MDH) [4,5]. Besides the two catalytic cysteines, the active site of Trx involves the conserved tryptophan residue adjacent to the most N-terminal cysteine and a buried aspartate residue that is conserved in all Trx sequences from bacteria to mammalian systems [6–8]. The redox potentials of Trxs are close to −300 mV, a value that makes them very efficient reductants of disulfide bridges [9]. The three-dimensional structure of Trxs is also very well described, all isoforms have a similar fold with a central pleated β sheet surrounded by α helices. The attacking cysteine as well as the active site tryptophan are surface-exposed while the other cysteine is more buried [10].

Grxs are rather well known in bacteria (such as *Escherichia coli*), yeast and mammalian systems. In *E. coli* there are three Grx genes, at least five in yeast and apparently only two in human [11–14]. In all known organisms, Grxs are reduced via a cascade that involves NADPH, glutathione reductase (GR) and the tripeptide glutathione [1]. The redox potential of Grx is estimated to be around −230 mV [15]. Although the targets of Grx are not as well characterized as those of Trx, it is quite clear that Grx is the preferred donor to ribonucleotide reductase and as efficient as Trx with PAPS reductase [16,17]. On the other hand, the information about plant Grxs is more scarce. The questioning of GenBank indicates that there are also multiple genes in plants with rather large variations at the active site (from the canonic YCPYC to the less frequent YCPFC, but also to more exotic forms as GCCMS where the active site sequence is hardly recognizable). Plant Grxs have been purified from several sources (rice and spinach mostly) [18,19]. The protein was found to possess activity in the 2-hydroxyethyl disulfide (HED) reduction assay and also in the more physiological dehydroascorbate (DHA) reduction [18]. Quite recently, Grx has been cloned and overproduced from poplar [20]. It has been shown to be very effective in the DHA reduction, but also to be a good electron donor to a new cytosolic peroxiredoxin (Prx) [21]. Quite interestingly, Trx, Grx and Prx have been found in sieve tubes and the phloem sap, sometimes in very large amounts, a property that suggests a role in the long distance transmission of the redox signal in plants [21–23]. One interesting feature of the poplar Grx is that it is elongated both on the N- and C-termini compared to the mammalian or bacterial Grxs characterized so far. It is thus of interest to investigate the biochemical reactivity of this type of protein in order to compare those data with the ones obtained with shorter versions of this protein in distant organisms. We describe in this paper a series of mutations that help understand the importance of the amino acids of the active site.

2. Materials and methods

2.1. Materials

Purified oligonucleotides, restriction enzymes, DNA polymerase and ligase were either from Eurogentec or from Invitrogen. IPTG,

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Abbreviations: DHA, dehydroascorbate; DTT, dithiothreitol; Grx, glutaredoxin; GR, glutathione reductase; HED, 2-hydroxyethyldisulfide; NADP-MDH, NADP-malate dehydrogenase; Prx, peroxiredoxin; Trx, thioredoxin

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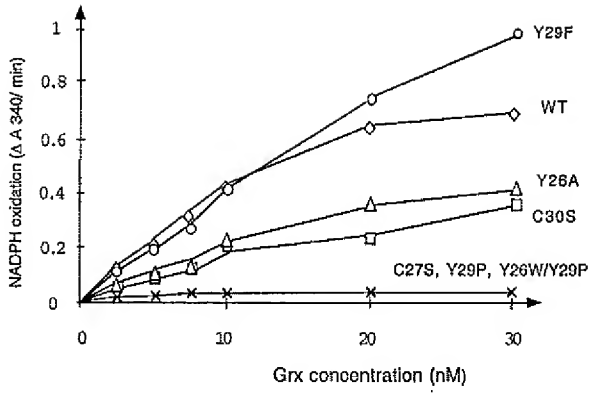

Fig. 1. Reduction of HED by WT and mutated poplar Grxs. All activities were measured at 30°C. After 1 min incubation of the system, the reaction was initiated by addition of Grx.

NADPH, HED, DHA, reduced glutathione (GSH) and GR were from Eurogentec, Boehringer, and Sigma. Chromatographic matrices were from Pharmacia. Kinetic measurements were followed using a Cary 50 spectrophotometer.

2.2. Plasmids and strains

The plasmid used for cloning and expression was pET-3d which carries the resistance for ampicillin. When needed, an additional plasmid (pSBET, carrying a kanamycin resistance) [24] was used to co-transform the expression strain *E. coli* BL21(DE3). The cloning strain was *E. coli* DH5α.

2.3. Mutagenesis

Mutagenesis of the cloned cDNAs was effected by PCR. A strategy similar to the one in [25] was employed, generating two overlapping mutated fragments and in a second PCR reaction the full length mutated sequence. The two 'cloning' oligonucleotides had the following sequences (*Nco*I and *Bam*HI restriction sites underlined): forward 5′-GGGCCATGGCTGGCAGCCCTGAAGCT-3′; reverse 5′-GGGGGGATCCTCATTCTAGTTTAAAGTCATC-3′.

The mutagenic oligonucleotides had the following sequences (mutagenic bases in bold): Y_{26}A FOR 5′-ATCTTCTCCAAGTCTGCTTGCCCGTATTGT-3′; Y_{26}A REV 5′-ACAATACGGGCAAGCAGACTTGGAGAAGAT-3′; Y_{26}W–Y_{29}P FOR 5′-TCCAAGTCTTGGTGCCCGCCTTGT-3′; Y_{26}W–Y_{29}P REV 5′-ACAAGGCGGGCACCAAGACTTGGA-3′; C_{27}S FOR 5′-TTCTCCAAGTCTTATAGCCCGTATTGTAAGAAG-3′; C_{27}S REV 5′-CTTCTTACAATACGGGCTATAAGACTTGGAGAA-3′; Y_{29}P FOR 5′-TCTTATTGCCCGCCTTGTAAGAAGGCT-3′; Y_{29}P REV 5′-AGCCTTCTTACAAGGCGGGCAATAAGA-3′; Y_{29}F FOR 5′-TATTGCCCGTTTTGTAAGAGG-3′; Y_{29}F REV 5′-CCTCTTACAAAACGGGCAATA-3′; C_{30}S FOR 5′-TATTGCCCGTATTCTAAGAAGGCTAAA-3′; C_{30}S REV 5′-TTTAGCCTTCTTAGAATACGGGCAATA-3′.

The template used was the construction pET-Grx3 [20]. After digestion with *Nco*I and *Bam*HI, the full length mutated fragments were cloned into pET-3d. The mutations were verified by DNA sequencing and the recombinant plasmids were used to transform the expression strain and then ampicillin and kanamycin resistant clones selected.

2.4. Expression and purification of the recombinant proteins

The transformed *E. coli* cells were successively multiplied to a final volume of ca. 5 l at 37°C. 100 μM IPTG was added in the exponential phase and the bacteria harvested by centrifugation for 15 min at 5000×*g*. The cells were resuspended in a TE buffer (Tris–HCl 30 mM, pH 8.0, EDTA 1 mM) which also contained 14 mM β-mercaptoethanol for the cysteinic mutants. All subsequent chromatographic steps were effected in the same buffer. The recombinant proteins were purified by ammonium sulfate fractionation (50–90%), Sephadex G50 gel filtration and DEAE Sephacel chromatography. The samples were then concentrated and dialyzed by ultrafiltration in an Amicon cell (Millipore) equipped with a YM 10 membrane under nitrogen pressure. The proteins were stored by aliquots, frozen at −20°C at concentrations of ca. 4 mg/ml. The yield was around 10 mg homogeneous protein per liter culture.

2.5. Biochemical assays

The assays describing the reductions of HED and DHA were performed as described in [20]. The Prx and NADP-MDH assays were as in [21,26]. The preparations of recombinant Grx, Prx and NADP-MDH have been described in [20,21,26].

The Prx reaction was effected in 500 μl cuvettes in the presence of 50 mM K-phosphate buffer, 150 μM NADPH, 1 mM GSH, 0.5 U GR, and 2.5 μM poplar Prx and Grx. The reaction was started by adding 100 μM H_2O_2 after 1 min of incubation at 30°C to permit the reduction of the system. Activity was measured by following the oxidation of NADPH at 340 nm.

The activation medium for NADP-MDH (30 μl) had the following composition: 50 mM Tris–HCl pH 8.0, 5 mM dithiothreitol (DTT), 0.8 μM recombinant sorghum NADP-MDH and 20 μM Grx or Trx as indicated. After 20 min incubation at 20°C, an aliquot of 20 μl was used to determine the activity at 30°C as described in [5].

The purity of the protein preparations was estimated by SDS–PAGE as described by Laemmli [27].

3. Results

3.1. Efficiency of the mutated Grxs in the 'classical' HED and DHA reduction tests

Fig. 1 shows the reactivity of the various mutants in the reduction of the non-physiological substrate, HED. Replacing Tyr 29 by a Phe residue had little effect on the activity, the protein behaving essentially as the wild-type (WT) enzyme. On the other hand, introducing a non-aromatic amino acid instead of either of the Tyr of the active site depressed the Grx activity (Y26A had a catalytic efficiency half the WT and the replacement of Tyr 29 by Pro resulted in an inactive protein). The C30S mutant retained 30% of the activity, but the C27S protein was inactive.

Essentially similar results were obtained in the DHA reduction (Fig. 2). When Tyr 29 was replaced by Pro, the protein was inactive and the replacement of Cys 27 by a serine likewise produced an inactive catalyst. Replacing Tyr 26 by an Ala decreased the catalytic efficiency (ca. 20%). On the other hand, the Y29F and C30S mutations produced better catalysts. It is especially remarkable that the monocysteinic mutant C30S which contains only Cys 27 is more efficient than the WT protein (the activity is nearly doubled at every concentration tested).

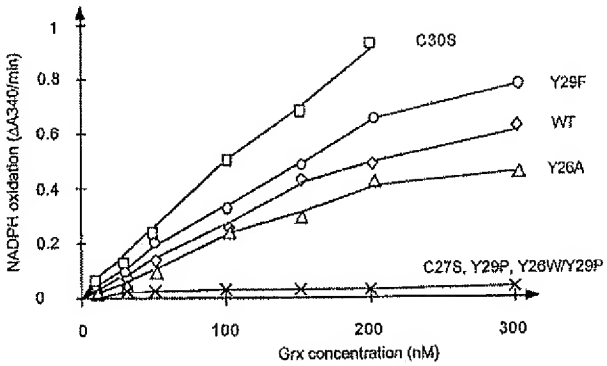

Fig. 2. Reduction of DHA by WT and mutated poplar Grxs. All activities were measured at 30°C. After 1 min incubation of the system, the reaction was initiated by addition of Grx.

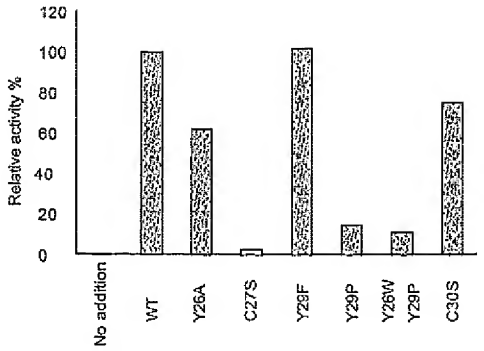

Fig. 3. Activity of Prx with WT and mutated Grxs. The activity is expressed in % of the WT protein. 100% activity is 0.55 ΔOD/min. Details of the reaction are given in Section 2.

3.2. Grx efficiency vs plant type C Prx

It has been demonstrated recently that type C Prx of plants uses both Trx and Grx as proton donors [21]. It was thus of interest to determine if the various mutants generated here are able to sustain the catalytic activity of Prx in this newly described reaction. Fig. 3 shows the results of those experiments. The WT and Y29F mutant show similar activity. On the other hand, replacing the two Tyr residues by non-aromatic side chains decreased the catalytic efficiency. Finally, the C30S mutant retains most of its catalytic efficiency and the C27S protein is inactive.

3.3. Activation of NADP-MDH by mutated Grxs

Two of the mutations that we have engineered in this work transform the Grx active site into a Trx-like active site (YCPPC and WCPPC). It was expected that these mutations should increase the reactivity of Grx vs the NADP-MDH, a well characterized target enzyme of Trx. Fig. 4 shows the reactivity of the various mutants with NADP-MDH. It is interesting that the WT Grx is able to activate the enzyme in the presence of DTT with reasonably good efficiency (nearly half the capacity of the non-physiological Trx *h* at the same concentration of 20 μM). All mutants tested showed catalytic activity, but all mutations decreased the efficiency except for Y26A. The two mutations that were intended to mimic the Trx active site (Y29P and Y26W/Y29P) did not improve the reactivity at all. Interestingly, both the C27S and C30S mutants retained the capacity to activate the NADP-MDH but their efficiencies were strongly reduced (to ca. 25% of the WT).

4. Discussion

4.1. Mutations of the active site cysteines

In the Grx dependent reactions (HED and DHA reduction and Prx activation), the mutation of Cys 27 leads to a virtually inactive enzyme. On the other hand, the mutation of Cys 30 into Ser had either a strongly negative effect (HED), a mildly negative effect (Prx) or a positive effect (DHA). All these data strongly suggest that Cys 27 is the primary nucleophile of the reaction and that Cys 30 is the backup cysteine. Similar results have been obtained with the *E. coli* and human Grx or all Trxs where the catalytic cysteine is always located on the N-terminus side [28–30]. It seems thus that the N- and C-termini extensions of plant Grx do not alter that property. Additionally, these site-directed mutagenesis experiments indicate that Grx can be quite efficient as a single cysteine catalyst, confirming that it can act in the so-called monothiol pathway [31]. Moreover, these experiments suggest that the numerous natural monocysteinic versions of Grx that exist in the databanks are almost certainly functional catalysts.

4.2. Importance of the aromatic residues of the active site

The replacement of the Tyr residue present between the two catalytic cysteines by a proline leads to a drastic decline in reactivity. It is clearly essential to keep an aromatic residue in this position as the Y29F mutant is a very good catalyst in all Grx dependent reactions tested. This Tyr residue has been implicated as one of the ligands necessary for the fixation of glutathione to *E. coli* Grx3 or T_4 Grx [11,32]. On the other hand, it is not necessary to have an aromatic residue in position 26 (N-terminus to the catalytic cysteine) as the Y26A mutant kept at least half of the reactivity of the WT protein. This is very much in contrast with the case of Trx where the removal of the Trp adjacent to the catalytic cysteine has a very strong negative effect [6,33].

4.3. The NADP-MDH is activated by plant Grx and its mutants

The NADP-MDH has been recognized as a target of Trxs since a long time. The chloroplastic Trx *f* is the most efficient, followed by chloroplastic Trx *m*, and the cytosolic Trx *h* [34]. Mammalian Trxs are very poor reductants/activators of this enzyme [35]. All Trxs which have been demonstrated as capable of activating this enzyme have either a WCGPC or a WCPPC active site. By making the Y29P and Y26W/Y29P mutations, we have engineered proteins with active sites that have been transformed into YCPPC and WCPPC. It was thus expected that these mutations could result in Grxs with better reactivity vs the NADP-MDH. The results in Fig. 4 indicate clearly that this is not the case as only the Y26A mutant has an activity comparable to the WT protein and all other mutations decrease the reactivity. It is surprising to observe that WT Grx is able to induce the activation of the enzyme in the presence of DTT (see Fig. 4). This activation is dependent on the concentration of added Grx and on DTT (data not shown). The WT poplar Grx is not as efficient as the cytosolic Trx *h* but more efficient than the human Trx or the Trxs of *Dictyostelium discoideum* [35,36]. We have checked that this reactivity is not due to contaminating *E. coli* Trx in the recombinant Grx preparations. We have indeed quantitated the

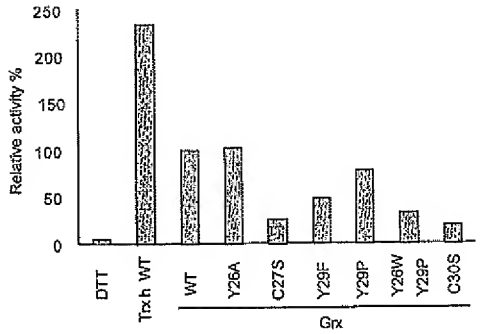

Fig. 4. Activity of NADP-MDH with WT and mutated Grxs. Activities are expressed in % of the WT Grx. 100% activity is 0.4ΔOD/min. Experimental details are given in Section 2.

contamination of those preparations by the NTR/DTNB system and found that it could not exceed 0.3%. At a Grx concentration of 20 μM in Fig. 4, the *E. coli* Trx concentration is thus lower than 60 nM, a value at which no detectable NADP-MDH activation occurs [37]. This activation of NADP-MDH by Grx is likely to be non-physiological since GSH could not replace DTT, even in the presence of NADPH and GR (data not shown). Three additional observations support the proposal that Grx is indeed not a physiological activator of NADP-MDH. First, it seems that there are no chloroplastic sequences for Grx in protein databases. Second, the redox potential of Grx (normally around −230 mV) is not adequate with respect to the ones of the NADP-MDH disulfide bridges (−280 and −300 mV) for an efficient reaction to take place [38]. Third, the fact that the C30S mutant keeps a level of activity similar to the C27S mutant suggests that the role of Grx in this reaction is non-catalytic. We propose thus that Grx acts here in a non-catalytic way to modify the accessibility of the active site to DTT which has the right redox potential. Such a structural role has already been observed in the case of the interaction between the T_7 DNA polymerase and the *E. coli* Trx [39]. The results obtained with the C27S mutant seem to support such a hypothesis since Cys 30 is widely recognized as the non-catalytic one, a property also verified in the other reactions described here.

4.4. Concluding remarks

Mutations similar to those described here have already been performed either on *E. coli* Trx to transform it into a Grx or a DsbA protein [40,41], or on DsbA to transform it into a Grx or a Trx [42]. In most of these mutants, essentially physicochemical characteristics as pK_a values and redox potentials have been determined but few kinetic experiments have been performed except in [40] where the creation of a CGHC site has been shown to be accompanied by an increase in PDI-like activity. In this study, nearly all the mutants that we have generated are less active than the WT protein, in contrast to initial expections. Transforming the Grx active site into a Trx-like active site did not improve the reactivity with NADP-MDH or Trx reductase (data not shown). Similarly to what is described here, simulations of Trx or Grx-like active sites in tryparedoxin 2 did not result in Trx or Grx-like activities [43]. Overall, these data strongly suggest that the structural determinants for Trx or Grx reactivity go beyond the sequence of the active site.

Nevertheless, the series of mutants generated here help to understand the functioning of plant Grxs. The catalytic cysteine as well as the importance of an aromatic residue inside the regulatory sequence and adjacent to the backup cysteine have been uncovered. An additional interesting finding of this study is the capacity of Grx to activate the NADP-MDH. This enzyme has always been reputed as promiscuous as it accepts many different Trxs as regulators, in contrast to fructose-1,6-biphosphatase that requires selectively Trx *f*. The data presented here indicate that the DTT NADP-MDH activation test should be used cautiously, as Grx is also active in this process despite the fact that it probably does not act in a catalytic way.

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